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**ISOLATION AND CHARACTERIZATION OF
CARBONIC ANHYDRASE FROM *OSTERTAGIA OSTERTAGI***

A Dissertation

**Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural Mechanical College
in partial fulfillment of the
requirements of the degree of
Doctor of Philosophy**

in

**The Interdepartmental Program in Veterinary Medical Sciences
through the Department of Pathobiological Sciences**

**by
Andrew A. DeRosa
B.S. Louisiana State University, 1992
M.S. Louisiana State University, 1997
August, 2004**

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ABSTRACT

The first event in the infection process of *Ostertagia ostertagi* in cattle is the process of exsheathment. Before trichostrongylid nematodes can transition from a free-living infective stage larva (L₃) on pasture to a parasitic existence within the ruminant host, it must first undergo exsheathment. Exsheathment is the process whereby the L₂ cuticle retained from the previous molt is cast from the L₃. Exsheathment enables the developmental transition from a free-living stage on pasture to a parasitic existence in the bovine host. For those species with a predilection site in the abomasum, such as *O. ostertagi*, exsheathment is initiated as the larvae pass through the rumen. Although the stimulus for exsheathment is not known, previously reported biochemical studies on exsheathment suggest the role of a carbonic anhydrase (CA). Partial support for this hypothesis comes from the reported failure of the *Haemonchus contortus* L₃ to exsheath following pretreatment with ethoxzolamide, a known inhibitor of CA's. Although convincing, a CA has not been previously reported from a trichostrongylid nematode. Therefore, the objective of this work was to isolate a CA gene from *O. ostertagi* L₃ and subsequently quantitatively measure its expression during *in vivo* exsheathment of *O. ostertagi* L₃. This work resulted in the successful isolation, cloning and sequencing of a gene that showed 90.5 % sequence identity with the CA eukaryotic consensus sequence and was 78% and 55% similar to the *Caenorhabditis elegans* cah-6 and human CAIII isozyme, respectively. This is the first CA isolated from a gastrointestinal nematode parasite. The enzyme was consequently named OoCAIII. The expression pattern of OoCAIII in *O. ostertagi* L₃ suggests this particular CA is not responsible for initiating exsheathment, but perhaps has a role in immediate early developmental events following initiation of exsheathment. Analysis of the first 1,758 bases of the proximal and

distal promoter regions of the gene suggested OoCAIII is regulated in part by transcription factors associated with hypoxic signaling and development.

INTRODUCTION

When left uncontrolled, parasitic gastrointestinal (GI) nematodes belonging to the taxonomic superfamily Trichostrongylidae can greatly reduce the efficiency of food animal production. Of these parasites, the brown stomach worm, *Ostertagia ostertagi*, is considered the most production limiting and economically important to cattle production enterprises in warm temperate and sub-tropical regions of the world. Although these climates allow for year around continuous grazing on pasture, the conditions are also more conducive for the survival of infective larvae (L₃) on pasture. Warm temperate and sub-tropical climates allow for a greater build up of L₃ on pasture, resulting in sustained exposure of cattle to these parasites, leading to higher infection levels and a greater potential for production losses in cattle.

Fortunately, dewormers (anthelmintics) with a high level of efficacy and persistent activity against GI parasites in cattle are available. These highly effective anthelmintics (ivermectin -Ivomec®, eprinomectin- Eprinex®, moxidectin-Cydectin®, doramectin-Dectomax®), however, belong to a single drug class, the macrocyclic lactones. In essence, the current luxury of highly efficacious and persistent control of all developmental stages of GI parasites in cattle is dependent essentially on this one class of drug. The development of wide spread resistance to macrocyclic lactones would have a pronounced negative effect on cattle production enterprises. This scenario has become a reality in the sheep industry, with a high global incidence of *Haemonchus contortus* resistance to macrocyclic lactone products. In cattle, case reports of macrocyclic lactone resistant strains of *Ostertagia* and *Cooperia* have been reported in South America and New Zealand (Coles 2002). Resistance and decreased efficacy to older dewormers such as the benzimidazoles (fenbendazole-Safeguard®, oxfendazole-Synantic®, and albendazole-Valbazen®) and imidazothiazoles (levamisole-Totalon®) has been

reported in four important worm genera in cattle (*Cooperia*, *Trichostrongylus*, *Haemonchus* and *Ostertagia*) (Sangster 2001).

Given the small pool of dewormers available for control of GI parasites, an emphasis should be placed on preserving the efficacy of existing dewormers and on discovering and developing dewormers that are diverse in both chemical structure and mechanism of action. The cost, however, of developing a new drug is considerable, with cost estimates exceeding US\$100 million, covering a time frame from discovery to market availability of 10 years (Witty 1999). In order to decrease both the cost and time line of discovery to launch, pharmaceutical companies have shifted from “whole organism” to “mechanism-based screening” for candidate compounds (Geary 2001), (Witty 1999). Mechanistic screening takes advantage of ultra high throughput robotic technology to screen hundreds of thousands of potential compounds for activity against a target molecule. However, the ultimate success of mechanism-based screening requires knowledge of fundamental parasite biology before a target molecule can be identified (Geary 1999).

Unfortunately, little is known about the biology of GI parasites of cattle. This void of knowledge, ironically, is due largely to the success of macrocyclic lactone dewormers. The effectiveness of these dewormers has resulted in the apparent devaluation of basic research on these important parasites by funding institutions such as government, universities, and the animal industry (Geary 1999). This need for renewed efforts to understand worm biology is echoed in a quote taken from a plenary paper presented at the 17th International Conference of the World Association for the Advancement of Veterinary Parasitology held in Copenhagen, Demark in 1999 by Dr. Timothy Geary, a well respected industry scientist in the field of anthelmintic discovery.

“Knowledge of the fundamental biology of helminths is woefully lacking; we comprehend very little of how these animals work as organisms. Investment in basic research in parasite biology will have an enormous impact on the development of new control strategies; indeed, without this investment, new anthelmintics may never be discovered. Figure out how worms work as organisms. Our ignorance of helminth biology is vast. The future of parasite control depends on developing a better understanding of the foe.”

Given this background, the impetus of the following work is to contribute to the understanding of the genetic basis for parasitism in trichostrongylid nematodes. More specifically, the aim is to determine how *O. ostertagi* (and perhaps other trichostrongylid nematodes) transition from a free-living (non-parasitic) life stage to a parasitic existence within its host. The experimental approach to this question intuitively is focused on the first event in the infection process, exsheathment.

Exsheathment of *O. ostertagi* within the rumen of its bovine host is required before the L₃ can develop to the first parasitic stage. Previous reports on the biochemical aspects of exsheathment of trichostrongylids from sheep suggest that the enzyme carbonic anhydrase (CA) plays a significant role in exsheathment. Although this biochemical evidence is convincing, a CA has not previously been reported from a trichostrongylid nematode.

Therefore, the specific goals of this work are to describe the kinetics of *O. ostertagi* exsheathment within the rumen, isolate a CA from this organism and describe its expression during the time course of exsheathment initiation and progression. The following series of chapters address these objectives. Preceding these chapters, however, an explanation of the form, function, and developmental stages common to trichostrongylid nematodes are provided in Chapter 1. Chapter 1 continues with a review of the literature pertaining to the biochemistry of

exsheathment of trichostrongylid nematodes. It also includes relevant information on carbonic anhydrases and known roles of these enzymes in mammalian systems.

The *in vivo* kinetics of *O. ostertagi* exsheathment is described in Chapter 2 as well as how the biochemistry of the rumen environment may function as a signal to the larvae to initiate development to the first parasitic stage. The potential role of the host diet in exsheathment is also discussed.

The isolation of a CA from *O. ostertagi* and a quantitative description of the CA expression pattern in *O. ostertagi* during the time course of exsheathment is the focus of Chapter 3. This work resulted in the first report of a CA from a trichostrongylid nematode. The expression pattern of this CA provides evidence for a developmental role of CA in *O. ostertagi*. The CA isolated from *O. ostertagi* had the greatest homology to the non-parasitic nematodes *Caenorhabditis briggsiae* and *C. elegans* CA, and to the human CAIII (HCAIII). Although a high homology exists between the *O. ostertagi* CA and the mammalian CA, the deduced amino acid sequences of *O. ostertagi* and *C. elegans* CA (cah-6) suggest the nematode CA's may possess unique structural differences.

As a first step in understanding the role of the isolated CA from *O. ostertagi*, Chapter 4 describes potential transcription activator binding sites located in 2kb of the sequence immediately flanking the 5' end of the CA gene (5'UTR). Transcriptional activator sequences isolated from the 5' UTR provided evidence for hormonal, hypoxic, and high-energy regulation of this CA. The presence of these sequences provides additional evidence supporting the hypothesis that this CA performs a developmental role in *O. ostertagi* following initiation of exsheathment.

I.1 REFERENCES

- Coles, G.C. (2002). Cattle nematodes resistant to anthelmintics: why so few cases? *Veterinary Research* **33**: 481-489.
- Geary, T.G., N.C. Sangster and D.P. Thompson (1999). Frontiers in anthelmintic pharmacology. *Veterinary Parasitology* **84**(3-4): 275-295.
- Geary, T.G. and D.P. Thompson (2001). *Caenorhabditis elegans*: how good a model for veterinary parasites? *Veterinary Parasitology* **101**(3-4): 371-386.
- Sangster, N.C. (2001). Managing parasiticide resistance. *Veterinary Parasitology* **12**(1-3): 89-109.
- Witty, N.J. (1999). Current strategies in the search for novel antiparasitic agents. *International Journal for Parasitology* **29**(1): 95-103.

CHAPTER 1

LITERATURE REVIEW

1.1 NEMATODE BASIC FORM AND FUNCTION

The phylum Nematoda includes some of the most abundant organisms on earth. Nematodes are typically bilaterally symmetrical, elongated, tapered at both ends, and possess a pseudocoel - a space between somatic muscles and the alimentary tract (Roberts 2000). These worms possess a complete digestive system with a mouth at the anterior end, an esophagus and an alimentary tract that runs the length of the body, composed of a single layer of cells, and an anus near the posterior tip of the body. The nematode body is completely covered with a noncellular cuticle that is secreted by cells on the surface of the hypodermis. The cuticle is shed four times during ontogeny. This shedding is a process referred to as molting and precedes the transition from one developmental stage to another. Beneath the cuticle lies a single layer of longitudinally arranged muscles that run the length of the body.

These three anatomical structures, the pseudocoel, cuticle and somatic musculature, function together as a hydrostatic skeleton that provides form to the organism and also acts as a platform of resistance from which locomotion is achieved. Coordinated motion and communication with the surrounding environment is accomplished through a simple yet complete nervous system consisting of two main elements: 1) the nerve ring located near the anterior end, 2) the dorsorectal ganglion and rectal commissure at the posterior of the organism. Dorsal and lateral nerve cords that span the length of the body connect these anterior and posterior elements.

1.2 ECOLOGICAL ASSOCIATIONS

Although morphologically similar, there is considerable biological and physiological diversity among nematode species. The diverse design of nematodes enables them to thrive in terrestrial and benthic environments as free-living organisms or as symbionts. In fact, the life

history of many nematodes includes both a free-living and symbiotic environment. One example of this is the gastrointestinal (GI) parasitic nematode of cattle, *Ostertagia ostertagi*. This organism completes three life stages on pasture herbage with subsequent symbiotic stages occurring within the GI tract of a bovine host. Symbiosis is defined as any two organisms living in close association, commonly one living in or on the body of the other (Roberts 2000). Symbiosis can be further classified as phoretic, mutualistic, commensal, or parasitic depending upon the nature of the interaction between the two organisms. An important feature of the Nematoda is that many species, such as *O. ostertagi*, are parasitic.

Parasitism is a symbiotic relationship whereby one participant, the parasite, either harms or lives at the expense of the other participant, the host (Roberts 2000). Just as free-living and non-parasitic symbionts can be found in virtually every ecological niche, parasitic nematodes can be found living within or on organisms belonging to virtually every phylum within the plant and animal kingdoms. Furthermore, several species are of great importance in the fields of human health agriculture and veterinary medicine.

1.3 THE NEMATODE CUTICLE

The extraordinary ability of parasitic nematodes to survive as free-living organisms in the external environment and then quickly acclimate and develop within their host is due largely to the design and function of the cuticle that covers their external surface and the buccal cavity, oesophagus, rectum, cloaca, vagina and excretory pore. Functionally, the nematode cuticle protects the body from injury, isolates the body from the external environment and retains body shape (Harris 1957). The cuticle also acts to provide locomotion. Nematodes do not have opposing muscles. Therefore, the elasticity of the cuticle, together with its high internal hydrostatic pressure (turgor), serves as a form of resistance likened to a water-filled balloon that enables the organism to straighten following muscle contraction (Kramer 1997). In the large

round worm, *Ascaris suum*, the resting pseudocolemic pressure has been measured at 70mm Hg above ambient pressure. It may reach as high as 225mm Hg above ambient pressure during movement (Pax 1995). Additionally, the cuticle plays a significant role in osmoregulation and ion regulation (Lee 1965), mediating nutrient uptake (Chen 1981) and waste excretion (Geary 1995) in some species.

Although ultrastructure of the *O. ostertagi* cuticle has not been studied in detail, the ultrastructure of the cuticle of *Haemonchus contortus*, a parasitic trichostrongylid nematode of sheep, and the non-parasitic model organism for modern nematology, *Caenorhabditis elegans*, has been described in great detail. Although the presence and thickness of each layer may be dependent upon the life cycle stage and species of the nematode, all nematodes have a layered cuticle (Lee 1965) consisting of 3 general layers; 1) the outermost epicuticle, 2) cortical region and 3) underlying basal layer. Bird and Bird (1991) further subdivided these three layers into 6 distinct layers or zones; 1) surface coat, 2) epicuticle, 3) cortical zone, 4) median zone, 5) basal zone and 6) hypodermis. The term ‘zone’ has been suggested as an alternate term to ‘layer’ because the regions sometimes merge into one another with no marked boundaries (Bird 1998). More recent examination of the *C. elegans* cuticle using freeze-etch electron microscopy has led to a slight refinement of the described layers. Based on this work, Kramer, 1997 (Kramer 1997) describes the six layers as 1) epicuticle, 2) cortical, 3) medial 4) fiber, 5) basal and 6) hypodermis.

The general structural composition of each cuticular zone, as typified by *C. elegans*, is as follows. A carbohydrate-rich glycocalyx, or surface coat (Blaxter 1992) 5 to 20nm thick, covers the epicuticle and is the interface of the nematode and its environment. The carbohydrates are present as conjugates, primarily in the form of glycoproteins and glycosphingolipids. Advances

in glycobiology research have led to an increased understanding of parasitic nematode glycoproteins particularly as potential targets for anti-nematode vaccines (Dell 2001).

The epicuticle is immediately below the surface coat. The epicuticle is a lipoprotein-rich trilaminar structure approximately 20nm thick. It forms the boundary between the external cortical layer and the outermost surface of the cuticle. Just beneath the epicuticle is the cortical layer. Amorphous and electron-dense, the cortical is composed largely of a highly insoluble protein cuticulin. Cuticulins were first described in *A. suum*, a roundworm of swine (Fujimoto 1973). These proteins, like collagens, have a high percentage of proline and glycine in their amino acid composition. Cuticulins, however, are resistant to bacterial collagenase, display a different X-ray diffraction pattern, and are characterized by a high degree of covalent dityrosine cross-linking that is thought to confer its chemical resistance to reducing agents such as sodium dodecyl sulfate and β -mercaptoethanol.

Filamentous fluid-filled columns of collagen, termed struts, form the medial layer and connect the cortical and fiber layers. The fiber layer consists of two layers of collagenous fibers that spiral around the animal in opposite directions. One layer runs clockwise and the other counterclockwise, approximately 65° from the long axis of the worm, forming a latticed matrix around the worm in a head to tail orientation (Kramer 1997). Beneath the fiber layer is the basement membrane, again composed of collagen fibers that merge with the underlying hypodermis.

The hypodermis is a syncytial layer with the nuclei contained in four thick portions known as hypodermal cords. Hypodermal cords project into the pseudocoel and longitudinally divide the somatic musculature into four quadrants (Roberts 2000). With an abundance of mitochondria and endoplasmic reticulum, the hypodermis has the important function of secreting the cuticular layers.

1.4 MOLTING

The life history of nematodes includes four molts with larval stages representing the intermolt periods (Any 1976). The molting process is defined by the cyclical synthesis of structural proteins, including collagens. For example, collagen mRNA levels are expressed in temporal waves corresponding with each molt during *C. elegans* development (Johnstone 1996). The high degree of organizational and structural homology that exists between collagen genes from *C. elegans* and collagen genes from *H. contortus* and *Trichostrongylus axei* suggests a similar cyclical pattern of gene expression may occur during molting in these, and other parasitic nematodes.

Three major steps, lethargus, late lethargus, and ecdysis occur during the molting process. Lethargus is characterized by a period of inactivity during which the old fibrous and basal layers and the hypodermis become separated. The hypodermis also becomes thickened and glandular, presumably due to the increase in the number of ribosomes and changes in the endoplasmic reticulum, golgi and mitochondria (Lee 1965). During late lethargus the new cuticle is formed (Page 2001). Throughout this process a stage is reached where the new and old cuticles are of equal thickness and are separated by large particle filled spaces. These particular spaces are presumed to function in the breakdown and absorption of the inner layers of the old cuticle (Lee 1965). The final stage of the molt, ecdysis, involves secretion of proteases that break down the old cuticle, leaving only the external cortical layer that is cast during the molt. The esophageal lining is replaced and the organism then pushes itself out of the old cuticle at the anterior end. In some species of nematodes, the old cuticle, consisting only of the external cortical layer is retained as a protective sheath for the larva. This sheath must be removed at some point through a process termed “exsheathment” (Any 1976) before it can progress to the next developmental stage. In the trichostrongylid nematode *O. ostertagi* it is this process that appears to determine

whether or not the organism will become parasitic following infection of its host (Rogers 1962).

1.5 *OSTERTAGIA OSTERTAGI*

When left uncontrolled, infections of parasitic gastrointestinal (GI) nematodes belonging to the superfamily Trichostrongyloidea can greatly reduce the efficiency of food animal production. Of the six families of parasites within the Trichostrongyloidea, the Trichostrongylidae contain one of the most pathogenic species of parasitic nematodes of cattle, *O. ostertagi*. *Ostertagia ostertagi* belongs to the Ostertagiinae – a subfamily of parasitic nematodes common to the abomasum or true stomach of ruminants.

1.5.1 Life Cycle and Epidemiology of *Ostertagia ostertagi*

Ostertagia ostertagi is considered the most economically important gastrointestinal nematode parasite of cattle in warm temperate climates and is one of three common trichostrongylid nematodes that infect the abomasum of cattle in the United States. The other two are *Trichostrongylus axei* and *Haemonchus placei*. Like all trichostrongylid nematodes, *O. ostertagi* has a direct life cycle consisting of three free-living stages, first stage larvae (L₁), second stage larvae (L₂), and third stage or infective larvae (L₃). Transition from the L₁ to L₂ requires a molting process whereby the L₁ cuticle is shed, exposing the newly formed cuticle of the L₂. As the L₂ develops to the L₃ stage, the L₂ larva also undergoes a molt. However, the L₂ cuticle, although separated from the underlying L₃ cuticle, is retained by the L₃. The retained L₂ cuticle completely encapsulates the L₃, preventing it from feeding. The L₃, approximately 0.6mm in length, subsequently migrates from the fecal pat onto surrounding herbage. The two cuticular layers enable the L₃ to survive for long periods on pasture herbage as it affords this stage a higher tolerance to desiccation and cold temperatures (Gibbs 1986).

After ingestion by a bovine host L₃, enter the rumen where the first event in the infection process begins, the process of exsheathment. Exsheathment is the process of shedding the

retained L₂ cuticle, exposing the underlying L₃ cuticle. The L₃ subsequently migrate to the abomasum where they burrow into the glandular tissue of the mucosal lining and molt to the early-fourth larval stage (EL₄) approximately four days following infection. EL₄ undergo further development by molting to the L₅ and reach sexual maturity 17 to 23 days post-infection. The mature or young adults, approximately 16mm in length, emerge from the glandular tissue to the gut lumen, mate, and subsequently begin egg production within the host.

Eggs are released into the lumen of the abomasum and subsequently dispersed into the outside environment through the host feces. Under ideal conditions, the eggs hatch within the fecal pat within 24hrs and rise to the L₁. The L₁ feed on bacteria within the fecal pat and develop to L₂ within 48hr to 96hr. Like L₁, L₂ feed on bacteria within the fecal pat. Both L₁ and L₂ are non-infective, free-living larval stages. L₂ larvae develop to L₃ within 9d to 14d following egg hatch. The L₃ is a non-feeding larval stage that migrates out of the fecal pat onto surrounding herbage and develops no further until it is ingested by a bovine host.

1.5.2 *Ostertagia ostertagi* Disease

Two types of ostertagiosis, Type I and II, are recognized in cattle based on the seasonal development of *O. ostertagi* (Williams 1971), (Williams 1987). Both forms result in similar and substantial destruction of the abomasal tissue as adult *O. ostertagi* emerge from gastric glands to the lumen. Type I ostertagiosis results from rapid acquisition of large numbers of L₃ that complete their development to the adult stage within the normal 3 to 4 week period. This occurs when environmental conditions are highly favorable to the survival and development of free-living stages. Type I ostertagiosis occurs during winter/early spring in southern temperate regions and during summer/early fall in northern regions of the United States. Type II ostertagiosis is associated with the maturation and emergence of previously developmentally arrested early-fourth stage larvae (EL₄). Developmental arrest of *O. ostertagi* occurs during the

late spring and summer months in southern warm temperate climates that experience extremely hot summers. It occurs during late fall and winter in northern cool temperate climates of the United States which experience extreme winter conditions (Williams 1971), (Williams 1987), (Gibbs 1986). Clinical outbreaks of Type II ostertagiosis generally occur during late summer/early fall in southern warm temperate climates and late winter/early spring in northern cool temperate regions of the United States. Common clinical manifestations of Type I and II ostertagiosis are anorexia, weight loss, diarrhea, hypoproteinemia, submandibular edema, and possible mortality (Williams 1987) (Gibbs 1986). However, a consensus among veterinary parasitologists is that subclinical parasitism has a greater economic impact on overall host welfare than clinical parasitism. This is due to effects such as reduced feed efficiency, altered or failed reproductive performance, inappetance, secondary infections, and other less well defined factors that contribute to overall loss of vigor (Gibbs 1986).

1.6 EXSHEATHMENT

Casting of the L₂ cuticle retained on the L₃ trichostrongylid larva marks the transition from the free-living to the parasitic stage and is described as the process of exsheathment (Sommerville 1957). While this process is apparently crucial to the development of parasitic nematodes, little is known about the physiology of the exsheathment process and no studies have been reported on the molecular basis of exsheathment.

Initiation of exsheathment has been studied extensively at the biochemical level in an attempt to discern the role of the host environment as a stimulus. Three suggested theories as to the role of the host in initiating exsheathment are 1) the host produces substances that cause exsheathment by their direct action on the sheath, 2) the host provides a substance that replaces a missing internal secretion that causes exsheathment, and 3) the host provides a stimulus that causes the infective larvae to produce internal secretions that act directly on the cuticle (Rogers

1960). Substantial evidence supports the third theory, suggesting that exsheathment is initiated through a stimulus or stimuli present within the host environment.

Evidence for rejecting the hypotheses that the host supplies a substance that acts directly on the nematode cuticle, or replaces a substance absent and required by ensheathed larvae, was provided by studies aimed at describing morphological changes in the cuticle following exposure to rumen fluid (Sommerville 1957). For example, in a series of studies *T. axei* larvae were mechanically removed from their sheaths. The empty sheaths were subsequently soaked in 38°C rumen fluid, to investigate the effect of the fluid on the removed sheath. As a control, live, sheathed, *T. axei* larvae were also incubated, separately, in the same rumen fluid. The criterion for determining initiation of exsheathment is the formation of a refractile ring around the sheath approximately 19µm from the anterior end of the larva (LaPage 1935), (Bird 1955), (Sommerville 1957), (Rogers 1960). There was no formation of this refractile ring in the empty *T. axei* sheaths bathed in rumen fluid. However, live larvae did form refractile rings on their sheaths and readily exsheathed in the rumen fluid. These data suggest that the action/activity that results in the formation of the refractile ring is initiated from within the live worm, not from outside of the cuticle.

A further study suggested that the active molecule(s) responsible for initiating exsheathment are actually secreted/excreted by the nematode. *Trichostrongylus axei* larvae were exsheathed in a cellophane sac within the rumen of a sheep. The supernatant was collected from these exsheathed larvae and subsequently added to mechanically removed sheaths from non-exposed larvae *in vitro*. As a control, a cellophane sac of water was left in the rumen and subsequently incubated with empty sheaths. The mechanically removed sheaths exposed to supernatant recovered from the exsheathed larvae formed refractile rings and the sheath split in

two layers as observed during normal exsheathment. Sheaths in control water showed no effect of treatment (Sommerville 1957).

Similar studies conducted by Rogers and Sommerville (Rogers 1960) demonstrated the production of an exsheathing fluid from *T. axei* and *H. contortus* larvae. In an effort to determine the location of exsheathment fluid production within larvae, *T. axei* larvae were ligated along various lengths of the body with a nylon ligature (Sommerville 1957). Results of these ligature studies suggested exsheathing fluid was produced within a 50µm region posterior to the excretory pore and anterior 25µm from the base of the esophagus (Sommerville 1957).

1.6.1 Composition of Exsheathing Fluid

Initial studies to investigate the composition of exsheathment fluid suggested it contained several of the same enzymes found in hatching fluids of nematode eggs (Barrett 1976) (Rogers 1958; Rogers 1965; Rogers 1977), as extracts from eggs and larvae act on the substrates of the other (Rogers 1982). One enzyme in particular, the zinc enzyme leucine aminopeptidase (LAP), was the focus of much research in this area. LAP has been implicated as the active enzyme in both exsheathing and hatching fluid, as both activities can be inhibited by the zinc protease inhibitor 1,10-phenanthroline (Rogers 1976). Furthermore, a LAP has been isolated from *Phocanema decipiens*, a GI nematode parasite of seals, during the molting cycle (Davey 1967), suggesting it may play an important role during this developmental process. However, other studies have failed to support a role for this enzyme in exsheathment. For example, mammalian LAP has been shown to be inactive against isolated *H. contortus* sheaths (Rogers 1965) and there has been some variability in the inhibitory ability of 1,10-phenanthroline on exsheathing fluid (Rogers 1976). Additional evidence against the LAP hypothesis came from Slocombe and Whitlock, (Slocombe 1974), (Ozerol and Silverman (Ozerol 1972) and (Slocombe 1969), who failed to isolate a LAP in exsheathing fluid in North American isolates of *H. contortus*.

To date, experimental evidence suggests a collagenase-like enzyme is responsible for the refractile ring formation and actual breakdown of the sheath during exsheathment. This collagenase-like enzyme, pseudocollagenase, is unable to break down native collagen; yet is active against the substrates Azocoll and p-phenylazobenzyloxycarbonyl (PZ)-L-Pro-L-Leu-L-Gly-L-Pro-D-Arg, comparable to the activity of purified collagenase from the bacteria *Clostridium histolytica* (Rogers 1982). Pseudocollagenase activity has been reported from exsheathment fluid. Indeed, in freeze-dried exsheathment fluid, pseudocollagenase was shown to be 400 times more active against isolated sheaths of *H. contortus* than the bacterial collagenase (Rogers 1982). Furthermore, the zinc inhibitor 1,10-phenanthroline was equally affective in limiting the activity of bacterial collagenase and exsheathment fluid on Azocoll and PZ L-Pro-L-Leu-L-Gly-L-Pro-D-Arg (Rogers 1982).

This observation reconciles earlier reports of inhibition of exsheathment by 1,10-phenanthroline where the activity of the inhibitor was thought to be against LAP (Rogers 1976). Interestingly, additional evidence for a nematode pseudocollagenase was supplied by earlier observations (Lewert 1956). In these experiments, *Strongyloides ratti* larval extracts were incubated with denatured and native collagen substrates. The *S. ratti* extract was active against denatured collagen, but had little to no activity against native collagen (Lewert 1956), suggesting a pseudocollagenase, versus true collagenase, activity.

The experimental evidence for the presence and activity of LAP and pseudocollagenase during the exsheathment process, and observations of Rogers (Rogers 1982), (Rogers 1960) suggest the following sequence of events leading to exsheathment. Larvae receive an extrinsic stimulus from the host upon entering the GI tract. This leads to neurosecretion and activation of an intrinsic factor such as LAP or a pseudocollagenase. LAP hydrolyzes cell membranes (Lote 1971) and therefore, may break down excretory cell membranes within the larvae. Breakdown of

the excretory cell membranes may lead to water uptake (Davey 1978) and the release of cell contents (Rogers 1982) such as pseudocollagenase, leading to refractile ring formation and subsequent exsheathment.

1.6.2 Artificial Exsheathment

Exsheathment can also, however, be initiated artificially by incubating larvae in a solution of 1.5% sodium hypochlorite (NaOCl) at 25°C. Exsheathment by artificial means appears to involve a mechanism separate from the processes set in motion by the host stimulus (Davey 1982). Davey, 1982, following the dynamics of water exchange in *H. contortus* during exsheathment, reported that *H. contortus* exsheathed by exposure to carbon dioxide (CO₂) lost 66pL of their total volume while exsheathment with NaOCl resulted in only a 54pL loss. Moreover, larvae exsheathed first with NaOCl and subsequently exposed to CO₂, lost an additional 20 pL of volume. These data were based on measurements of ³H₂O uptake of larvae (Davey 1982). In an effort to identify the source of volume loss, Davey and Sommerville, (Davey 1982) implemented quantitative interference microscopy to measure changes in the optical path difference (OPD) of various regions of the *H. contortus* larvae following exsheathment in CO₂ at 38°C and NaOCl (Davey 1982). The OPD is the measure of light passing through an object compared to the medium in which the object is immersed. For a biological sample, the opd is proportional to the concentration of materials in the sample. Thus, changes in the OPD reflect changes in the concentration of materials. Moreover, if changes in concentration are not a result of synthesis of additional material, then changes in opd will be inversely proportional to changes in volume (Davey 1982). The authors observed that larvae exsheathed with CO₂ and NaOCl lost relatively the same volume, 19 and 22 pL respectively, from the esophagus. However, the opd between the excretory cells of unstimulated and CO₂ - stimulated larvae were significantly different. This difference represented an increased density

and, therefore, a decreased volume, of excretory cells in CO₂-stimulated larvae. There was no significant difference observed, however, in the opd of excretory cells between NaOCl-stimulated and unstimulated worms.

The authors concluded that two parallel processes were being set in motion by stimulation with CO₂ at 38°C and that only one of these processes was mimicked by exposure to NaOCl (Davey 1982). Based on these data it was suggested that CO₂ may act at more than one site, or that it stimulates a single receptor element that in turn stimulates two target organs via different routes (Davey 1982). To further study the exsheathment process, then, it was necessary to either replicate the host environment in an *in vitro* system, or utilize the parasite host in direct studies.

1.6.3 *In Vivo* and *In Vitro* Exsheathment

First reported attempts to discern the stimulus for exsheathment were made by incubating infective *H. contortus* larvae in solutions of HCl and NaOH in the absence of rumen fluid. Initiation of exsheathment of *H. contortus* using this approach required an 8-day incubation period at 38°C (LaPage 1935). Similarly, infective *Trichostrongylus retortaeformis* larvae (a trichostrongylid stomach parasite of rabbits) exposed to a solution of HCl and pepsin, led to exsheathment after 60hrs of incubation (Crofton 1947). While this was a major advance in understanding the biology of these nematodes, this system did not appear to mimic the timing of the exsheathment process *in vivo*. In the 1950's, however, Sommerville (Sommerville 1954) and Bird (Bird 1955) both demonstrated that *H. contortus* larvae would exsheath if placed within the abomasum of a sheep in a cellophane dialysis sac. Sommerville (Sommerville 1957) subsequently reported that initiation of exsheathment was more efficient in the region of the GI tract immediately anterior to the predilection site of each parasite. In this study, he reported exsheathment rates of trichostrongylid species with predilection sites of the abomasum (*H.*

contortus, *T. axei*, *O. circumcincta*), small intestine (*T. columbriformis*, *Nematodirus spathiger* and *N. abnomarlis*) and large intestine (*Oesophagostomum columbianum*) following *in vivo* and *in vitro* exposure to the rumen, abomasum and small intestinal content. Placement of *H. contortus*, *T. axei* and *O. circumcincta* directly into the rumen or in extracted rumen fluid maintained at 38°C resulted in rapid exsheathment. This was later confirmed by Rogers and Sommerville (Rogers 1960), who showed that both *H. contortus* and *T. axei* larvae exsheath faster following *in vivo* implantation compared with *in vitro* exposure to rumen fluid.

Other studies have also shown that the predilection site of a parasite affects its ability to exsheath under specific conditions (Sommerville 1957). For example, *H. contortus*, *T. axei*, and *O. circumcincta* failed to exsheath following *in vivo* exposure to the abomasum or small intestine of a fistulated sheep, but did exsheath when exposed *in vitro* to sheep abomasal fluid artificially maintained at 38°C. In contrast, the small intestinal species *T. columbriformis*, *Nematodirus spathiger* and *N. abnomarlis* failed to exsheath following implantation in the rumen, or following *in vitro* exposure to rumen fluid. They did, however, readily exsheath in the abomasum and abomasal fluid. It is interesting to note that the average pH of the ovine and bovine abomasum is 4.0, compared to a pH of 5.0 to 7.0 for the rumen. These observations suggest that intestinal parasitic nematodes require acidic environments for initiation of exsheathment.

One species that challenges this theory is *Cooperia curticei*. This trichostrongylid parasite of the bovine small intestine had a higher rate of exsheathment when exposed to rumen fluid, relative to rates following exposure to the abomasum (Ahluwalia 1974). Interestingly, the predilection site of this parasite is the pylorus, which is adjacent to the abomasum, suggesting the mechanism for exsheathment of *C. curticei* is more similar to abomasal parasites rather than species that establish further into the small intestine.

1.6.4 Stimuli for Exsheathment Initiation – Carbon Dioxide and Bicarbonate Ions

In 1960, Taylor and Whitlock reported that *H. contortus* larvae would exsheath in a media derived from anerobically grown *Aerobacter aerogenes* (Taylor 1960). Further analysis of the system revealed that the minimum effective components of the system were 0.5 percent solutions of several salts (NaCl, KCl, NaBr and NaHCO₃), into which free CO₂ had been bubbled (Taylor 1960). The necessary components of the system were media saturated with CO₂ and a temperature not less than 37°C. However, considerable variation in exsheathment was observed using different salts. The authors suggested this variation was due to the ability of these salts to catalyze the reaction of water (H₂O) and CO₂ to undissociated carbonic acid (H₂CO₃), so that $\text{CO}_2 + \text{H}_2\text{O} \rightarrow \text{H}_2\text{CO}_3$. They also suggested, but did not test, the role of pH in the process.

In the same year, it was reported that exsheathment initiation in nematode larvae was related primarily to the concentration of H₂CO₃ and/or dissolved gaseous CO₂ (Rogers 1960). Further analysis of the components of this reaction ($\text{CO}_2 + \text{H}_2\text{O} \rightarrow \text{H}_2\text{CO}_3 \rightarrow \text{H}^+ + \text{HCO}_3^-$) were tested and it was concluded that the concentration of undissociated H₂CO₃ was important in initiating exsheathment (Rogers 1960).

Data supporting this was obtained by exposing *Trichostrongylus axei* and *H. contortus* larvae to phosphate or sodium bicarbonate-carbon dioxide buffers with and without 0.02M sodium dithionite (reducing agent) (Taylor 1960). Gas mixtures consisting of nitrogen and 1, 2.5, 5, 10, 20, and 40% CO₂ were bubbled into the buffer solutions and concentrations of sodium bicarbonate were added to produce a pH 6.0, 6.9, 7.3, and 8.0. All experiments were carried out in 0.05 M NaCl solutions at 37°C. A maximum of 6% and 3% exsheathment of *T. axei* and *H. contortus* larvae, respectively, occurred in the phosphate buffered solutions, regardless of CO₂ concentration. However, greater than 50% exsheathment of *T. axei* occurred at 5% CO₂ in bicarbonate buffer that yielded a pH of 6. In contrast, only 14% of *H. contortus* larvae

exsheathed under the same conditions and lower rates were observed at all other treatment levels (Rogers 1960). Results of these experiments revealed that sodium bicarbonate, bicarbonate and carbonate ions alone did not initiate exsheathment, but some activity could be obtained if CO₂ was introduced.

Based on these observations it was deduced that the concentration of undissociated carbonic acid and/or dissolved gaseous CO₂ were responsible for inducing exsheathment. Furthermore, it was suggested that the requirements for exsheathment of *H. contortus* were more rigorous than *T. axei* (Rogers 1960). Indeed, a dose response was observed between increasing concentrations of undissociated H₂CO₃ and exsheathment rate for both nematode species in 40% CO₂. Maximum exsheathment (81%) of *T. axei* occurred at 1.0×10^{-3} M H₂CO₃ at pH 7.3. For *H. contortus*, maximum exsheathment of only 34% was achieved at 0.6×10^{-3} M H₂CO₃ while exsheathment rates of 60% were observed under conditions of 6.0×10^{-3} M H₂CO₃ and pH 6.0. Similar results were obtained when CO₂ levels were increased to 100%. Addition of the reducing agent, sodium dithionate, generally increased the rate of exsheathment for both species and the relative increase in percent exsheathment due to the reducing agent was always greater at lower concentrations of H₂CO₃. The effect of the reducing agent also increased as the oxidation-reduction potential of the solution decreased.

Following their conclusion that H₂CO₃ and / or dissolved gaseous CO₂ were responsible for initiating exsheathment, the authors further suggested that factors such as pH, oxidation-reduction potential (Eh) and reducing agents affect exsheathment indirectly, by either enhancing or decreasing the effect of the two main components (Rogers 1960). Effects of osmotic pressure were also explored using sodium chloride titration of bicarbonate-CO₂ buffers and found to have a negligible affect on exsheathment rates (Rogers 1960). Similar results were reported by Taylor,

1960 whereby osmotically equivalent solutions of salt and sugar had no significant effect on exsheathment rates in *H. contortus* (Taylor 1960).

1.6.5 Stimuli for Exsheathment – Ph and Redox Potential

The pH of the environment has also been shown to affect exsheathment of parasitic nematodes, as suggested by Taylor, 1960. Exsheathment of *T. axei* and *H. contortus* larvae was optimal in rumen fluid within the pH range of 6 to 6.5. Alternatively, exsheathment rates were higher within the range of pH 3.5 to 4 for the intestinal nematode *T. columbriformis* (Rogers 1960). These values correlate with the pH of the GI compartment where exsheathment occurs. For *H. contortus* and *T. axei*, exsheathment is most efficient in the rumen, which has a normal pH of 6.0 to 6.5. Exsheathment of *T. columbriformis*, a small intestinal nematode, occurs in the abomasum where a pH 4 is normal. As reported by Taylor (Taylor 1960), optimal exsheathment rates of *T. axei* and *H. contortus* both occurred at temperatures of 37°C. Exsheathment was not initiated, however, at temperatures below 25°C or above 58°C.

In addition to pH and temperature, the authors also observed that the oxidation-reduction potential of the media had a marginal effect on exsheathment rates. Exsheathment rates generally increased as the potential fell. As with the effect of the reducing agent, the oxidation-reduction potential of the culture media had a more pronounced effect on the exsheathment rate of *T. axei* larvae compared to *H. contortus* larvae. In this study the authors also noted that pH, a reducing agent and temperature were necessary for stimulating the exsheathment process, but alteration of these factors after initiation did not prevent the process from proceeding.

1.6.6 The Interaction of CO₂, Ph and Redox Potential In Exsheathment

There is much evidence to suggest that the stimuli for exsheathment act in conjunction with one another. For example, Slocombe and Whitlock (Slocombe 1969) showed that CO₂, pH, salt concentration and temperature were all important in the initiation of exsheathment of *H.*

contortus. In this experiment, *H. contortus* larvae were suspended in .02M sodium tetraborate, gassed with 40% CO₂/60% nitrogen, incubated at 37°C in a water bath shaker for 20min, and then gassed for 5 min with compressed air to remove the CO₂. Under these conditions larvae exsheathed rapidly. The same conditions were also applied to the infective larvae of *Nematospiroides dubius*, a small intestinal nematode of mice, in an effort to induce exsheathment (Cypress 1973). After an adjustment of the sodium tetraborate concentration from .02M to .01M, and a longer post-gassing incubation period from 20min to 30min, *N. dubius* larvae readily exsheathed *in vitro* with exsheathment rates reaching 89%. These authors also reported that exsheathment rates increased as the number of larvae per suspension increased. These observations are presumed to be due to the increased concentration of exsheathment enzymes in solution and support the hypothesis that increasing larval concentration increases exsheathment rates due to the increased concentration of exsheathment fluid in the media (Silverman 1964). The differences in the optimal conditions for exsheathment between *N. dubius* and *H. contortus* may reflect the differences in the GI environments (pH, CO₂, and salt concentrations) in which each of the species exsheath.

Similarly, initiation of exsheathment of *Labiostrongylus eugenii*, a strongylid nematode stomach parasite of the Kangaroo Island wallaby (*Macropus eugenii*), was also dependent upon CO₂, pH and temperature. The effect of the reducing agent, sodium dithionate, however, was minimal, even at low partial pressures of CO₂ where its effect is most pronounced on *H. contortus* (Smales 1976). *Labiostrongylus eugenii* exsheaths in the sacculated portion of the wallaby stomach which has an environment similar to the rumen of ruminants. Exsheathment rates of *L. eugenii* larvae were greatest in 0.85% sodium chloride media, with sodium bicarbonate added to pH 7 at 35°C, gassed with 40% CO₂ for 1hr and subsequently aerated for 24hr. Exsheathment did not occur at 30°C and at CO₂ concentrations of 5% or less. Based on the

conditions of the media, the optimum concentration of undissociated H_2CO_3^* and dissolved gaseous carbon dioxide for exsheathment was calculated to be $2.0 \times 10^{-3} \text{M}$ (Smales 1976). This concentration is higher than the optimal range ($0.1 \times 10^{-3} \text{M}$ to $0.6 \times 10^{-3} \text{M}$) reported for other trichostrongylid nematodes (Rogers 1960).

Sensitivity to the exsheathment stimulus may differ greatly between nematode species depending on the GI compartment in which exsheathment occurs. This is exemplified by the responses of *N. dubius* and *H. contortus*. *Nematospiroides dubius* will readily exsheath when incubated in a solution of 0.01N HCl and a CO_2 concentration of 0.033%. While *H. contortus* is insensitive to HCl concentrations as high as 2N and will exsheath only when total CO_2 concentrations exceed 5% with maximum exsheathment occurring at 40% CO_2 (Petronijevic 1986). These two species represent extremes in their sensitivity to CO_2 stimuli for exsheathment. Similar responses of nematode species whose predilection site is the small intestine, such as *T. columbriformis* and *Nematodirus battus*, have been reported to exsheath more efficiently when exposed to concentrations of HCl and low pH and thus exsheath in the abomasum.

Rogers and Sommerville (Rogers 1960) reported the effect of rumen fluid in promoting exsheathment of *T. axei* and *H. contortus* was made inactive by mild treatment such as centrifuging, filtering, freeze drying, or boiling. Based on these observations the authors suggested at least two unstable factors, one being a reducing agent, were important in initiation of exsheathment. For *T. axei*, the activity of the rumen fluid could be restored with the addition of a reducing agent (1 mg/ml sodium dithionite), although the results varied from 5% to 100% reactivation with different samples of rumen fluid. Restoration of activity, however, was not observed for *H. contortus*, suggesting the requirements for exsheathment are more rigorous for this species.

1.6.7 A Role for Carbonic Anhydrase In the Initiation of Exsheathment

Amalgamation and review of the experimental data on larval exsheathment strongly suggests that some component of the system $\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{H}_2\text{CO}_3 \rightleftharpoons \text{H}^+ + \text{HCO}_3^-$ is essential in the stimulus for exsheathment over the pH range of 3.0 to 8.0; especially the total concentration of $\text{CO}_2 + \text{H}_2\text{CO}_3$ (Petronijevic 1985). Within this system, low partial pressures of CO_2 are effective at low pH values as demonstrated for *N. dubius*. This was evidenced by the observation that at pH 3.0 the concentration of HCO_3^- was negligible. Furthermore, the concentration of H_2CO_3 was $1.9 \times 10^{-8} \text{M}$ (pH 2.0, 0.033% CO_2). The nematode *N. dubius* was induced to exsheath under these conditions (Petronijevic 1985), (Sommerville 1973). *Haemonchus contortus* also exsheathed at pH 2.0 when exposed to higher H_2CO_3 concentrations ($2.3 \times 10^{-5} \text{M}$) in the presence of increased CO_2 (40%) (Petronijevic 1985). At higher pH values, where H_2CO_3 may be low (e.g. $4.9 \times 10^{-7} \text{M}$), a greater concentration of HCO_3^- would provide adequate amounts of H_2CO_3 . Bicarbonate alone was shown not to induce exsheathment in *N. dubius* and *H. contortus* and it was difficult to distinguish the effect of CO_2 and undissociated H_2CO_3 (Petronijevic 1985). These data support those of Rogers, 1960 (Rogers 1960) whereby bicarbonate and carbonate ions alone failed to induce exsheathment.

(Petronijevic 1985) has suggested that the rate of exchange within the system, rather than the concentration of the components and the rate of exchange within the larva itself, may be important factors in stimulating exsheathment. The important product of this exchange within the larvae is thought to be the production of H^+ ions, leading to a rapid decline in internal pH (Petronijevic 1985). The action of the enzyme, carbonic anhydrase, would support the theory of a CO_2 sensor, as suggested following assessment of *H. contortus* larvae with interference microscopy.

Evidence for the role of carbonic anhydrase in exsheathment was also reported by Davey (Davey 1982). Effects of a carbonic anhydrase inhibitor, ethoxzolamide, on the two putative parallel processes of exsheathment (Davey 1982) were explored. Only 3% of *H. contortus* larvae incubated in 2×10^{-5} M ethoxzolamide for 12h, and subsequently stimulated with CO₂ at 38°C, exsheathed. Conversely, 73% of untreated larvae exsheathed under the same conditions. Similar observations were previously reported by Rogers, 1980, where carbonic anhydrase-specific inhibitors, ethoxzolamide and acetazolamide, blocked exsheathment of *H. contortus* (Rogers 1980). Interestingly, treatment with ethoxzolamide inhibited the increase in opd of excretory cells normally observed upon stimulation with CO₂ at 38°C, but did not appear to have any effect on the esophagus (Davey 1982). Iodine, a reversible inhibitor of carbonic anhydrase, also yielded results comparable to the effects of ethoxzolamide. Data from these experiments confirmed earlier observations of two parallel processes following CO₂ stimulation leading to exsheathment. One of these processes appeared to occur in the esophagus and the other in the excretory cells. The data also showed that ethoxzolamide inhibits the process leading to the release of exsheathing fluid from excretory cells, strongly suggesting the role of carbonic anhydrase in initiation of the exsheathment process (Davey 1982).

1.6.8 Exsheathment and Larval Development

Petronijevic and Rogers, (Petronijevic 1983) suggested that successive developmental stages of nematodes (embryo, four larval stages and adult) are controlled by sets of genes appropriate to each stage of development and each set is turned on at appropriate points in the life cycle. The authors further suggest that in species with a simple life history, such as soil dwelling, non-parasitic nematodes with a relatively constant environment, the threshold for switching on the gene sets would be low so that development would appear to be continuous. However, when development of different stages occurs in very different environments, the

threshold for switching on a gene set governing development in the new environment would be increased. The stimulus characteristic of the new environment would be required to switch on the appropriate gene set. Therefore, for parasitic nematodes with life cycles that are partly free-living and parasitic, the gene set of the parasitic stage carried by the infective stage would be switched off until the stimulus from the host indicated that a suitable environment had been reached.

It is likely that the stimuli that invoke the induction of a set of parasitic genes are concentrations of CO₂ and undissociated H₂CO₃^{*}, and the efficiency of this induction is probably dependent upon a complex relationship between CO₂, Eh, cation concentration and temperature. In an attempt to discern whether exsheathment is controlled by the gene set for the first parasitic stage the following questions have been asked: 1) does the stimulus lead to transcription of genes before exsheathment occurs or, 2) are the genes required for exsheathment transcribed in the preceding L₂ life stage?

To investigate these questions, sheathed *H. contortus* L₃ were exposed to 8x10⁻⁷M and 8x10⁻⁶M actinomycin-D (to block transcription) and stimulated with 42% CO₂ at 39°C to initiate exsheathment. Following exposure to stimulating conditions for exsheathment, larvae were transferred to media and conditions conducive for development to the fourth-larval (L₄) stage, the first parasitic stage. The percentage of L₃ in both groups exposed to actinomycin-D exsheathed at a rate comparable to non-treated L₃. However, only 7% of the L₃ exposed to 8x10⁻⁶M actinomycin-D developed to the early L₄, while greater than 60% of untreated L₃ and L₃ exposed to 8x10⁻⁷M actinomycin-D subsequently developed to the early L₄ (Petronijevic 1983). Similarly, exposure of L₃ of *Nippostrongylus braziliensis* (Bonner 1976), a nematode parasite of rodents, and *Neoaplectana glaseri* (Desponsmmer and Jackson 1972) a nematode parasite of insects, to 8x10⁻⁶M and 2.5x10⁻⁵M actinomycin-D respectively, prevented further development of these parasites to the first parasitic stage. However, exsheathment was apparently unaffected

by exposure to these concentrations of actinomycin-D as both species readily exsheathed both *in vitro* and *in vivo*. These observations suggest that the genes governing exsheathment are transcribed during the L₂ stage but the genes required for development to the first parasitic stage are controlled at the transcriptional level in the L₃ following receipt of a stimulus from the host (Petronijevic 1983).

1.6.9 Hypoxia, Exsheathment, and Larval Development

Based on the CO₂-rich rumen environment and biochemical studies of the *in vitro* response of L₃ to simulated rumen conditions, the host stimuli(s) appear(s) to be either a high CO₂ concentration, temperatures above 34°C, hydrogen ion concentration, undissociated H₂CO₃⁻, bicarbonate, or the complex interaction of all of these factors. These observations correspond to the environment of a normal functioning rumen where the gaseous composition of the rumen content is 65% CO₂, 27% CH₄, 7% nitrogen, with trace amounts of H₂S, CO, and transient quantities of O₂. The normal rumen temperature is 38°C-40°C, pH is diet dependant (4.5 to 7.0), and there is a high H₂CO₃⁻ / bicarbonate concentration (Dehority 2003).

The mechanisms by which these factors may activate the exsheathment process is not known, although biochemical studies suggest carbonic anhydrase plays a significant role (Petronijevic 1983), (Davey 1982), (Rogers 1977), (Rogers 1960), (Sommerville 1957), (Sommerville 1954). Additionally, links between hypoxia (oxygen concentration < 3%) (Duranteau 1998), carbonic anhydrase activity and subsequent cell signaling events leading to regulation of gene expression and development have been described in other metazoan species. The remaining sections will review the literature pertaining to hypoxic signaling and the molecular byproducts of carbonic anhydrase activity and their roles in initiation of gene expression in other metazoan organisms.

1.7 SIGNALING IN RESPONSE TO HYPOXIA

1.7.1 Hypoxia Inducible Factors

Cellular adaptation to hypoxia has been studied extensively in the past decade. Many processes involved in oxygen homeostasis are mediated by hypoxia-inducible factors (HIFs) that regulate the transcription and expression of several dozens of target genes. HIF's, therefore, represent the link between oxygen sensors and effectors at the cellular, local, and systemic levels (Wenger 2002). HIF's were first discovered during DNA-protein interaction studies aimed at understanding the regulatory mechanisms of erythropoietin (Wenger 2002). In this study a hypoxia response element (HRE) [TACGTGCA] was identified on the 3' end of the erythropoietin gene. Several trans-acting factors bound to the HRE, one of which bound exclusively following hypoxic exposure (Bunn 1996). This DNA binding factor was subsequently named HIF-1 or hypoxia inducible factor 1 and is a phosphorylation-dependent and redox-sensitive protein that contacts DNA in the major groove (Semenza 1992).

HIF-1 is a heterodimeric protein composed of a 120kDa α and a 91-94kDa β subunit (Wang 1995). Upon initial isolation and sequence analysis of the two subunits, it was discovered that HIF-1 α was a novel protein and the HIF-1 β subunit was identical to the previously identified heterodimerization partner of the dioxin receptor/aryl hydrocarbon receptor (AhR), called AhR nuclear translocator (ARNT) (Hoffman 1991). Subsequent cloning and complete sequencing of HIF-1 α cDNA revealed that HIF-1 α belonged to the same class of proteins as ARNT, containing a basic helix loop helix (HLH) DNA binding domain and a common domain called PAS. PAS is an acronym for the first three members of this family, namely Per/ARNT/Sim (Gu 2000). The PAS domain serves as an interface for protein-protein interactions.

1.7.2 Regulation of HIF-1 α Subunits

HIF-1 α is a constitutively produced protein that is regulated in part by an oxygen-dependent degradation domain (ODD) that is covalently modified by hydroxylation through the activity of HIF-prolyl hydroxylases (HPH's) at the carboxyl-terminal trans-activation (TA) region (Jaakkola 2001),(Ivan 2001),(Huang 1998). HPH's are dioxygenases. They require oxygen and 2-oxoglutarate as co-substrates and contain iron bound by two histidine and one aspartic acid residues. The HPH's transfer an oxygen atom onto the proline residues of the HIF- α subunits, forming a hydroxyl ion on proline. A second oxygen atom reacts with 2-oxoglutarate yielding succinate and CO₂ as products.

The hydroxylation of the HIF- α residues stabilizes the protein but does not fully activate it. Under oxygen deprived conditions, or when the HPH's are inactivated by competitive substrate analogs, the HIF- α prolines remain unmodified (Epstein 2001). Inactivation of HIF- α occurs when the hydroxylated proline residues initiate a modification of an asparagine residue. These hydroxylated asparagines interfere with recruitment and binding of the coactivator CBP/p300 (Lando 2002).

Hydroxylation of the HIF- α ODD domain confers rapid degradation of the protein through the proteasomal degradation pathway, by enabling the binding of the protein von Hippel Lindau (pVHL) to the hydroxylated ODD domain. The pVHL is part of an ubiquitin ligase linking the HIF- α subunits via elongin C to a complex composed of several proteins to the ubiquitinylation machinery. Cells deficient in functional pVHL show constitutively high HIF- α subunit levels and expression of many oxygen-regulated genes. Consequently, HIF- α subunits are rarely isolated from normoxic cells (Huang 1998).

HIF- β (ARNT) is similar to HIF- α in that it is constitutively transcribed. However, unlike HIF- α , the ARNT subunit is not regulated by hypoxia. ARNT serves as a heterodimerization partner of PAS proteins such as HIF- α . Formation of the HIF-1 α complex (HIF- α + ARNT) is essential for DNA binding and trans-activation. This complex is formed when HIF- α translocates to the nucleus and binds with ARNT to form HIF-1 α . The HIF-1 α heterodimer has two TA domains, one overlapping the ODD domain and the other at the carboxyl terminus. These two TA domains confer transcriptional activation of target genes primarily by recruiting transcriptional co-activators such as CBP/p300 (Wenger 2002). CBP/p300 is a histone acetyltransferase recruited by several transcription factors. Histone acetylation modulates chromatin structure to open the genomic locus for increased transcriptional activity (Gu 2000).

Upon chromatin remodeling the HIF-1 α transcription factor binds to the consensus HIF-1 α DNA binding site (HBS) [A/G CGTG] present in the HRE's of many oxygen-regulated genes (Camenishch 2001). An HBS is necessary but not sufficient for efficient hypoxic gene activation, other DNA binding sites for additional transcription factors are required. The interplay between these additional transcription factors and HIF-1 α requires binding of the cotranscription factor CBP/p300 (Wenger 2002).

1.7.3 Regulation of Gene Expression Through HIF's

Molecular oxygen is essential for survival of mammals due to its critical role as an electron acceptor during ATP production via oxidative phosphorylation in the mitochondria and because several enzymatic processes require oxygen as a substrate (Wenger 2000). Consequently, hypoxia serves as a physiological stimulus that affects all organisms in unique ways (Ganesh 2004). Similar to intracellular energy status, cellular oxygen concentration is

closely regulated in mammals to maintain cellular function and integrity. Reduced oxygen availability initiates a series of adaptive responses that are by HIF's. These HIF's trans-activate several target genes whose products function to increase oxygen delivery and to enhance metabolic adaptation to anaerobic conditions (Ganesh 2004).

Many of the physiological responses result in expression of genes encoding for proteins responsible for glycolysis, the anaerobic production of ATP. Such genes include aldolase A, enolase- α , lactate dehydrogenase, pyruvate kinase, and glucose transporter-1. In addition, some well-known target genes of HIF's are those encoding vascular endothelial growth factor (VEGF), erythropoietin, glucose transporters and glycolytic enzymes, which stimulate angiogenesis, erythropoiesis, and glycolytic ATP production, respectively (Minyoung 2003). Other gene targets include those encoding growth factors, stress proteins, apoptotic proteins, antioxidants, and protein kinases and phosphatases (Wenger 2002). In most cells examined, HIF's also enhance the synthesis and accumulation of stress-related proteins such as several members of the heat shock proteins (HSP) such as HSP90, HSP70, and HSP27 (Graven 1993). Also, cells exposed to chronic hypoxia promote the expression of Nip3, a member of the bcl-2 family of cell death factors (Bruick 2000).

Hypoxic conditions also alter circadian rhythms in mice through the function of HIF-1 α . HIF-1 α was shown to stabilize the circadian rhythm PAS protein PER1 through protein-protein interactions following induction of hypoxia. In addition, HIF-1 α was also shown to form a heterodimeric transcription factor with another known circadian rhythm protein CLOCK. Thus, HIF-1 α can regulate circadian rhythms by posttranslational modification and transcriptional regulation of circadian rhythm proteins and genes, respectively (Dmitri 2001).

1.7.4 HIF-Mediated Developmental Signaling

Hypoxia often serves as an important HIF-1 α mediated developmental signal. For example, during placenta development, hypoxia maintains trophoblast proliferation and prevents differentiation toward an invasive phenotype. In addition, knockout mice lacking HIF-1 α expression within the cartilaginous growth plate of the developing bone show gross skeletal malformations and die perinatally, due apparently to tracheal abnormalities. In these mice, cells in the hypoxic interior of the growth plate die as result of defective HIF-1 α growth arrest (Wenger 2002).

The role of HIF-1 α in mediating developmental processes by regulation of cellular proliferation and differentiation can be explained largely by its role in signal transduction pathways that promote cell survival and development. Under hypoxic conditions, the activation of several stress and mitogen activated protein kinases (MAPK) has been demonstrated in different cell types and is associated with the activation of transcription factors such as activator proteins (AP-1, c-jun, c-fos, Fra-1, ATF-2), elk, and nuclear factor- κ B (NF- κ B) (Bilton 2003). A good example of HIF-1 α MAPK activation leading to cell survival is in rat PC12 pheochromocytoma cells. PC12 cells can be differentiated into neural cells following stimulation with neural growth factor. Exposure of these cells to moderate hypoxia (6% O₂) strongly activated both the p38 α and p38 γ isoforms of the p38/SAP kinase 2 pathway. ERK was also activated to a lesser extent. ERK is a MAPK associated with cell survival. When PC12 cells were incubated for 6hr under severe hypoxic conditions (1% O₂), ERK1 activity was highly upregulated. In addition, exposure of PC12 cells to hypoxia caused membrane depolarization, extracellular calcium influx and activation of several calmodulin kinases and cAMP responsive element binding protein (CREB) (Conrad 1999).

A downside of HIF1- α activity is the role of these proteins in producing an environment for malignant cell survival and proliferation leading to tumorigenesis through the formation of an acidic extracellular and temporal intracellular environment. Maintenance of an acidic extra and intracellular environment is accomplished through: 1) upregulation of glycolysis where pyruvate is converted to lactic acid 2) upregulation of carbonic anhydrases resulting in increased intracellular hydrogen ion and bicarbonate production and 3) down regulation of sodium/hydrogen exchangers (NHE) and sodium-bicarbonate cotransporter proteins, the results of which downregulates the acid-extruding capacity of the cell leading to a decrease in intracellular pH (Wenger 2002), (Wenger 2000), (Wykoff 2000).

It is well documented that mammals use both systemic and cellular strategies to adapt to decreased oxygen levels during normal development and homeostasis. Hypoxic tissues secrete growth factors such as vascular endothelial growth factor (VEGF) to increase vascularization, and individual cells increase anaerobic metabolism to sustain basic cellular functions. Transcriptional mediation of these local and systemic responses is largely under the control of HIF-1 α in mammals (Minet 2001).

As with mammals, invertebrate species also encounter hypoxic conditions either during developmental processes or within their natural environment, suggesting, therefore, a need for an oxygen sensing mechanism. The environment of the soil dwelling nematode *C. elegans* is often hypoxic and this organism has been shown to adapt to very low oxygen levels. Further investigation into the role of hypoxic adaptation resulted in the isolation and sequencing of both HIF- α and ARNT from *C. elegans*. Given these findings, nematode species may in fact utilize similar cell signaling pathways and consequently similar gene expression patterns for adaptation to hypoxia (Huaqi 2001).

There is considerable evidence in mammalian systems that hypoxic conditions can stimulate a signaling cascade that leads to upregulation of genes required for cellular development, differentiation, or alteration of metabolic function. Given these observations and the finding of ARNT and HIF-1 α homologs in *C. elegans*, it is not unreasonable to hypothesize a role of hypoxia as a developmental “switch” for trichostrongylid nematodes. How larvae might sense the low O₂ environment in the rumen is not known, but the activity of a carbonic anhydrase could function to facilitate rapid diffusion of CO₂ across cell membranes. Activity of a carbonic anhydrase coupled with the activity of HIF-prolyl hydroxylases could theoretically result in a rapid initiation of a hypoxic signaling cascade and perhaps function to trigger developmental and metabolic genes required for the next parasitic stage.

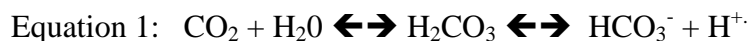
It has been suggested that carbonic anhydrases play a role in initiation of exsheathment in the trichostrongylid nematodes *H. contortus*, *T. circumcincta*, and *T. axei* (Petronijevic 1985), (Davey 1982), (Rogers 1960). Based on the role of carbonic anhydrases in mammalian systems, there are a number of ways carbonic anhydrase activity could function as a signal to the larvae that it is in a host environment. The following sections introduce the carbonic anhydrases and present a brief explanation of some of the functions of each active carbonic anhydrase reported from mammalian systems.

1.8 MAMMALIAN CARBONIC ANHYDRASES

1.8.1 Classification

The enzyme carbonic anhydrase (CA) was discovered in 1933 in bovine erythrocytes as the result of a search for a catalytic factor that would increase the transfer of bicarbonate from the erythrocyte to the pulmonary capillaries (Meldrum 1933). Since the discovery of the first CA, now known as CA I, numerous CAs have been discovered in almost all living organisms examined. CA's are classified into three genetically unrelated families of isoforms (α , β , γ) and

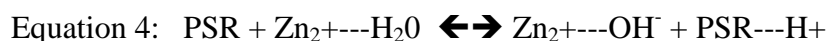
share very little sequence identity between the classes. The α and γ class enzymes are strictly monomers and trimers, respectively, and members of the β class are dimers, tetramers, hexamers, and octamers (Chegwidden 2000). Despite their gross structural differences, the active sites of all three classes utilize a single bound zinc atom for catalysis of the reversible hydration of CO_2 resulting in the interconversion of CO_2 and bicarbonate (Equation 1). The reaction is initiated by the addition of acid to a bicarbonate solution, producing CO_2 and consuming H^+ , or by adding CO_2 to an alkaline solution, consuming CO_2 and producing H^+ . This reaction is fundamental to a variety of physiological processes that involve pH homeostasis, CO_2 and HCO_3^- transport and water and electrolyte balance (Tripp 2001). Exemplary roles of CA's in mammalian systems include renal and male reproductive tract acidification, bone resorption, respiration, gluconeogenesis, signal transduction, net acid/base transport, and formation of gastric acid. More recently, some CA isoforms have been related to cell proliferation and oncogenesis. (Breton 2001).



1.8.2 Catalytic Mechanism

Kinetic studies indicate that all three classes of CA's employ a two-step, "ping-pong" mechanism to carry out the reversible hydration of CO_2 . This mechanism was deduced through extensive study of the activity of the α -class of CA's. The first step involves the nucleophilic attack of a zinc-bound hydroxide ion on CO_2 (Equation 2), yielding a bicarbonate ion. The active site hydroxide ion is regenerated in the second step by ionization of the zinc-bound water molecule and removal of a proton from the active site (Equation 3). The zinc ion functions as a Lewis acid to lower the dissociation constant (pK_a) of the water molecule from ~ 14 to 7.0 (Tripp 2001), (Khalifah 1991).

Most CA's have catalytic rates (kcat) greater than 10^4 s^{-1} . This high rate of catalysis requires one or more proton shuttle residues (PSR) (Equation 4) to transfer the proton from the metal-bound water molecule to the external buffer (Equation 5). Consequently, the rate-limiting step for CA's and other enzymes with such high kcat is the transport of protons from the metal ion in the active site to buffer molecules in solution. The kinetics, however, of this mechanism are quite variable depending on conditions such as pH, and substrate and buffer conditions.



The molecular basis for variation in the catalytic rate observed between the various CA isoforms has been largely explained through comparative analysis of the crystal structures and respective amino acid composition of five isoforms of α -CA's (Tripp 2001), (Khalifah 1991).

1.8.3 Carbonic Anhydrase Inhibitors

It is within the rate-limiting step of the CA enzyme that the classical CA inhibitors (acetazolamide, methazolamide and ethoxzolamide) prevent CA function; at least with the α -CA's isolated from mammals and invertebrate species (Lindskog 1997). These compounds are heterocyclic sulfonamides that bind metal ions. Thus, the sulfonamide anions bind directly to the zinc ion in the active site of the CA, resulting in a perturbation of the CA catalytic cycle by preventing the proton shuttle process. CA's in plants, however, are apparently insensitive to sulfonamide inhibition. The sensitivity of mammalian CA's to sulfonamide inhibitors has contributed greatly in determining the physiological function of CA isozymes in cells, organelles, and solutions.

1.8.4 α -Carbonic Anhydrases

Currently, 14 isoforms of α -CA's have been discovered, although only 11 of these are active (CAI – CA VII, CA IX and CA XII – CA XIV). The remaining three (CA-VIII, CA X, CA XI) are inactive due apparently to substitutions of two or more histidine residues required to bind zinc, which is essential for the activity of CO₂ hydration. These proteins, due to their lack of enzymatic activity, have been described as CA related proteins (CARPs); the functional significance of these proteins is not known (Chegwidden 2000). All α -CA's discovered thus far are monomeric zinc-metalloenzymes of approximately 29kDa molecular weight and are collectively pleiotrophic enzymes, as indicated by their wide tissue and sub-cellular distribution. A brief description of each mammalian CA is included below. The sub-cellular distribution of each active α -CA is illustrated in Figure 1.1.

1.8.4.1 Carbonic Anhydrase I

Until the early 1960's CA was thought to exist in one form. At this time, however, two distinct molecular forms were isolated from human and bovine erythrocytes, with different enzymatic activities, and were termed CAI and II (Lindskog 1960). The catalytic rate of human CAI based on the hydration of CO₂ and the dehydration of bicarbonate at 25°C is $2 \times 10^5 \text{ m sec}^{-1}$, which is considerably slower than the $1.4 \times 10^6 \text{ m sec}^{-1}$ of CAII. Functionally CAI contributes to approximately 50% of CO₂ hydration in mammalian erythrocytes and is thought to be a redundant isoform as long as CAII is present.

This presumed redundancy is based on the absence of CAI in red cells of ruminants and the fact that individuals who are homozygous for human CAI deficiency exhibit no related clinical abnormalities (Khalifah 1991). CAI has also been isolated in surface epithelial cells of the colon, cryptal enterocytes of the small intestine, corneal endothelium, capillary endothelium,

placenta and fetal membranes and eye lens of mammals. The primary function of CAI in intestinal tissues of mammals appears to be the cycling of bicarbonate and hydrogen ions, pH homeostasis, and water and ion transport (Khalifah 1991).

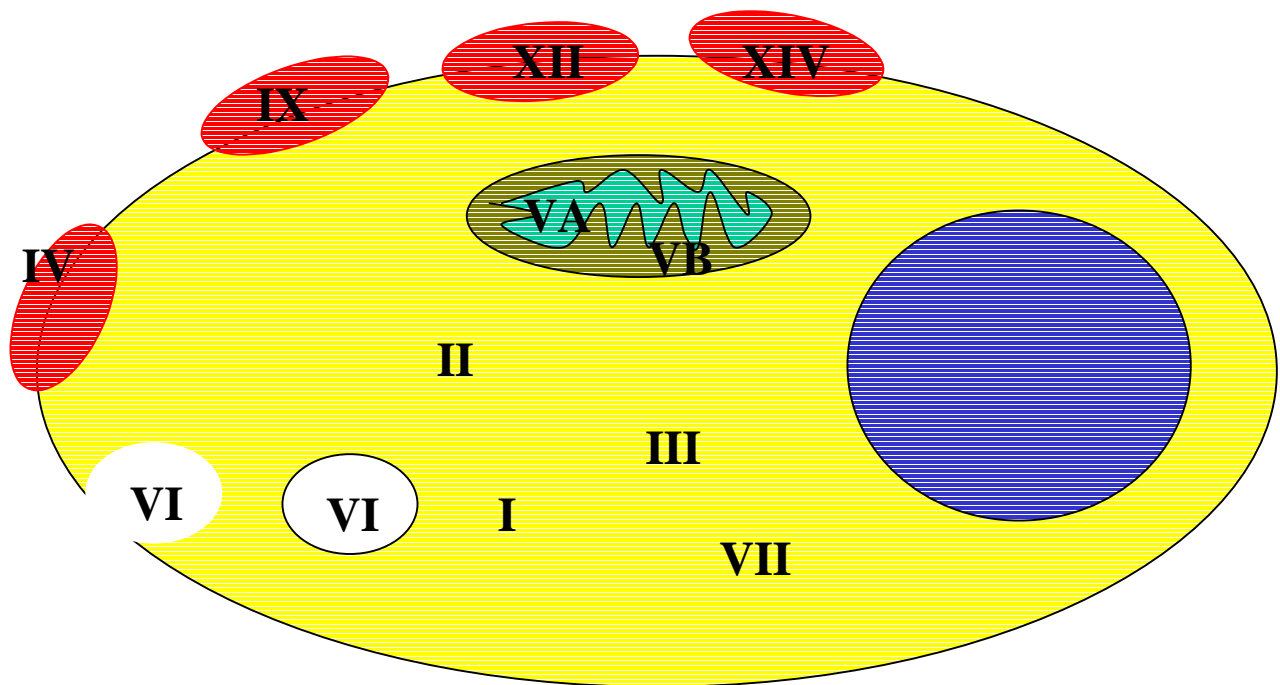


Figure 1.1. Subcellular location of active carbonic anhydrase isoforms in mammalian cells. Loss of expression of CAI in normal colorectal mucosa in humans is often associated with progression to malignant cell transformation (Kivela 2001).

The genomic architecture of CAI consists of 7 exons and, including the 5' and 3' untranslated sequences, consists of 50kb of sequence. Based on analysis of expressed sequence tags (EST's) and Northern analysis of reticulocyte RNA, there appears to be alternative splicing within the 5' region of the gene. However the physiological significance of this 5' splicing is not known (Butterworth 1991). In addition to transplicing, mRNA transcripts in erythroid and non-erythroid tissues show that human CAI gene is controlled by two promoters separated by 36kb of DNA sequence, and with different tissue specificities (Brady 1991). In human erythroid cells the

CAI gene is developmentally regulated, with expression occurring late in fetal development (~40wks), at about the time of the switch from fetal to adult globin gene expression.

1.8.4.2 Carbonic Anhydrase II

Carbonic anhydrase II (CAII) is the fastest enzyme known with a turnover rate of $1.9 \times 10^6 / \text{s}$ under physiological conditions. It was first found in erythrocytes, where it is involved in hydration of CO_2 (Wistrand 1981). CA II is expressed in the cytoplasm of cells in most organs of the digestive system and is thought to release bicarbonate ions in the saliva. In the liver, CAII has been demonstrated in hepatocytes and epithelial cells of the bile ducts, where it produces bicarbonate for alkalization of bile. CAII is also expressed in epithelial cells of the gallbladder, where it is involved in bile concentration and acidification (Parkkila 1996).

1.8.4.3 Carbonic Anhydrase III

CAIII differs from other CA isoforms by having a lower specific activity (~ 1% of CAII) and is resistant to the CA inhibitor acetazolamide. CAIII constitutes 8% of the soluble protein in red skeletal muscle and up to 25% of that of differentiated adipocytes (Cabiscol 1996). CAIII is negligible in preadipocytes, becoming substantial upon differentiation. An ELISA for CAIII concentrations in canine serum has been developed to monitor muscle tissue disorders in dogs (Nishita 2002).

Although it is highly abundant in fat and muscle, the function of CAIII is not fully understood. It has been implicated, however, in fatty acid metabolism, where it may facilitate rapid conversion of glycolytic intermediates to oxaloacetate and citrate and stimulate their incorporation into fatty acids. CAIII has two reactive sulfhydryl groups that can conjugate to glutathione through a disulfide link in a process termed S-glutathiolation. CAIII is rapidly glutathionylated *in vivo* and *in vitro* when cells are exposed to oxidative stress. This observation suggests that CAIII may play a role in cellular response to oxidative stress. In fact, analysis of

protein expression patterns in protein deprived mouse liver revealed that CAIII and copper and zinc superoxide dismutase proteins were greatly reduced, indicating a diminished anti-oxidant response (Sanllorenti 2001).

1.8.4.4 Carbonic Anhydrase IV

CAIV was the first membrane-associated CA isoform described (Whitney 1982). It is anchored through a glycosylphosphatidylinositol link to the apical plasma membrane of epithelial cells (Zhu 1990). CAIV has been isolated from humans and rats, where it is associated with colonic and small intestinal epithelia. The luminal plasma membrane of the human gall bladder and bile duct epithelium express large amounts of CAIV in close association with CAII and membrane Na/H exchangers. These observations suggest a complementary system for bile concentration and acidification. CAIV is developmentally regulated in mouse placenta where its expression triples from day 11 to day 15 and then remains stable until the end of pregnancy (Rosen 2001).

1.8.4.5 Carbonic Anhydrase V

CAV has a unique localization to the mitochondrial matrix (Nagao 1993) and has been isolated from liver and pancreas tissues of humans and rodents (Parkkila 1997). CAV has very low CA activity, but plays a role in the mitochondrial processes of ureagenesis and gluconeogenesis. It provides bicarbonate ions for the first urea cycle enzyme carbamyl phosphate synthetase I, and for the first step in gluconeogenesis where pyruvate carboxylase converts pyruvate into oxaloacetate (Heck 1994).

1.8.4.6 Carbonic Anhydrase VI

CAVI is the only known secretory isoform of CA and is normally found in mammalian salivary glands and saliva (Parkkila 1990). The function of CAVI was first thought to be the maintenance of bicarbonate levels in the saliva (Fernley 1988). However, it was later shown not

to have a direct role regulating pH or buffering of saliva, but rather functions in the enamel pellicle, which is a thin layer of proteins covering the teeth. Here the enzyme may provide protection to teeth by neutralizing acid produced by cariogenic bacteria (Kivela 1997). Additionally, CAVI is thought to contribute to the maintenance of the pH gradient in the stomach by accelerating the neutralization reaction that produces CO_2 and H_2O .

1.8.4.7 Carbonic Anhydrase VII

CAVII like CAI, II, and III is a cytoplasmic enzyme and possesses only about 4% of the CA activity of CAII. The amino acid sequence of CAVII is the most highly conserved CA isoenzyme in mammals (Earnhardt 1998) CAVII has been isolated from the human salivary gland, rat and mouse lung and mouse brain. This isoform is also developmentally regulated in the rat. CAVII expression emerges abruptly within the hippocampus of the 12d postnatal rat where it functions as a molecular switch in generating high frequency stimulation induced GABAergic excitation. These observations suggest CAVII activity may contribute to susceptibility to epileptogenesis (Ruusuvuori 2004).

1.8.4.8 Carbonic Anhydrase IX

CAIX was first recognized as a novel tumor-associated antigen expressed in human cervical carcinoma cell line HeLa. Expression of this isoform is mediated in part via promoter/enhancer elements at the transcriptional level. Characterization of the CAIX promoter region revealed a functional HRE in positions -3/-10 upstream of the transcriptional start site. CAIX regulation has been demonstrated to occur through the trans-activation of HIF-1 α through binding at the HRE in HeLa carcinoma cell lines (Loncaster 2001). The expression of CAIX protein is positively regulated by cell density and ectopic expression in NIH3T3 cells promotes cell proliferation. These cells lose capacity for growth arrest and grow chaotically on top of one another with a 15% faster doubling time and a 10% enhancement of DNA synthesis. This

isoform also acidifies the extracellular milieu surrounding tumor cells and in this promote tumor growth and spread (Ivanov 2001). For these reasons CAIX has been postulated as an intrinsic marker of tumor hypoxia.

1.8.4.9 Carbonic Anhydrase XII

Like CAIX, CAXII is highly expressed in cancer cells and was first identified in a renal cell carcinoma (Tureci 1998). Also like CAIX, this isoform is regulated transcriptionally by the trans-activity of HIF-1 α during hypoxia. This isoform is expressed in normal tissues of the kidney, intestine, prostate, pancreas, testis, ovaries, brain, and lung. Two transmembrane isoforms of carbonic anhydrase, CAIX and CAXII, are highly expressed in hypoxic tumor cells. This isoform also appears to function in acidification of the extracellular environment (Ivanov 2001).

1.9 NEMATODE CARBONIC ANHYDRASES

Although α CA's have been isolated from a number of invertebrates (Henry 1984), the only nematode species from which α CA gene has been isolated and sequenced is *C. elegans*. These sequences were identified as a result of the *C. elegans* genome sequence project. A total of 6 *C. elegans* α CA isoforms are available at www.wormbase.org and are listed in Table 1.1. Only 4 of the 6 are active CA isoforms, and of the active forms, all of them have high sequence similarity with the human CAVII. There is no literature available on the developmental expression or function of these CA isoforms within the organism.

1.10 POTENTIAL INTERACTIONS OF HYPOXIA AND CARBONIC ANYDRASE IN NEMATODES

1.10.1 Signaling Through Hydrogen Ions (Cellular Acidosis)

Working on the assumption that trichostrongylid nematodes possess an active CA, this enzyme could serve as part of the exsheathment mechanism. It can be hypothesized that the CO₂ rich environment of the rumen would provide a substantial amount of substrate for CA within the

Table 1.1. Summary of carbonic anhydrase isoforms isolated from *Caenorhabditis elegans*.

<i>C. elegans</i> CA gene	Exons	Amino Acids	Percent identity with human CA	Human CA homolog
cah-1	6	365	36%	CARP X
cah-2	9	337	36%	CARP X
cah-3	4	246	97.2%	CAVII
cah-4	6	280	77.9%	CAVII
cah-5	6	309	80.9%	CAVII
cah-6	5	319	76.2%	CAVII

nematode as CO₂ readily diffuses across the nematode cuticle and would be available for membrane bound or cytosolic isoforms of CA (Barrett 1976). The high concentration of CO₂ substrate for CA would result in a rapid increase in intracellular hydrogen and bicarbonate ions, the byproduct of rapid hydration of CO₂ by CA. A rapid increase in intracellular bicarbonate and hydrogen concentration can potentially serve as stimuli for expression of genes related to development (Deitmer 1996). It has been demonstrated in human erythrocytes and in HEK293 cells that CAII binds to the C-terminal tail of the AE1 bicarbonate/chloride anion exchanger. The CA/AE1 complex minimizes the distance bicarbonate must diffuse prior to efflux and minimizes the local concentration of bicarbonate during influx mode. The rapid efflux of bicarbonate coupled with the rapid influx of CO₂, and subsequent hydrogen production through the activity of CA, leads a rapid decrease in intracellular pH (temporal cellular acidosis) (Sterling 2001).

In neurons, rapid pH transients are not simply a mere result of a perturbation of acid-base regulation, but act as cell signals. Studies on the central nervous systems of both vertebrates and

invertebrates have shown that neuronal activity leads to defined extra- and intracellular pH changes. These consist of mono and multiphasic shifts, indicating that they might originate from multiple sources and/or via multiple pathways (Deitmer 1996). A summation of studies on membrane polarization of leech glial cells and associated shifts in intracellular pH concluded that, considering H^+ transients as signals, CO_2 , bicarbonate, and CA would be essential elements in this signaling. These would determine, then the shape of the pH shifts in time and space within nervous systems (Deitmer 1998). A similar pathway may be used in the chemosensory mechanism of *C. elegans* larvae as they exit the dauer stage. In *C. elegans*, metabolic activation is accompanied by a large decrease in intracellular pH from 7.3 to 6.3 within 3hrs after dauer larvae encounter bacteria. This shift occurs before feeding begins, and it coincides with, or soon follows, the commitment to resume development (Riddle 1997). Like the dauer larvae of *C. elegans*, the infective third stage larva of trichostrongylid nematodes is also a developmentally arrested stage and may rely on a similar intracellular signal for resumption of development to first parasitic stage upon entry into its host.

Transition from aerobic to anerobic metabolism in the intestinal nematode *Ascaris suum*, requires the establishment of the phosphophenolpyruvate carboxykinase-succinate pathway (PEPCK). This transition occurs during the molt from the L_3 to the L_4 stage. PEPCK is a gluconeogenic enzyme that catalyzes the first committed step of gluconeogenesis (Kita 1997). Expression of this enzyme in mammalian kidney cells is increased several fold under metabolic acidosis through *cis* enhancer elements and trans acting factors. For example, the promoter region of PEPCK contains binding sequences for the transcription factors NF- κ B, AP-1, and CREB (Krewnowska 2002). Expression of these transcription factors is mediated by HIF-1 α activation during hypoxia. In fact, the components and organization of the respiratory chain

change dramatically in *A. suum* larvae during the transition from L2 to adult as the respiration shifts from aerobic to anaerobic metabolism (Kita 1997).

1.10.2 Signaling Through Bicarbonate Ions

Bicarbonate is another potential intracellular signaling mechanism and is a byproduct of CA activity within a hypoxic environment. Bicarbonate ions are powerful inducers of the second messenger soluble adenylyl cyclase (sAC). sAC synthesizes the cell signaling molecule cyclic adenosine mono phosphate (cAMP). cAMP mediates several cellular effects by activating other cell signaling molecules, such as cAMP protein kinase (PKA, RAP exchange proteins, and cAMP gated ion channels) (Zippin 2003). PKA is anchored to the mitochondria by A-anchoring proteins (AKAP) located on the membrane surface of mitochondria. Consequently AKAP tethered PKA on the mitochondria suggests that sAC may be a source of cAMP mediating PKA-dependent phosphorylation of the pro-apoptotic protein BAD and/or PKA mediated inhibition of cytochrome c oxidase (COX) activity. COX oxidizes reduced cytochrome c using oxygen as a terminal electron acceptor, and produces H₂O during aerobic respiration. In this way, bicarbonate will reflect changes in CO₂ through perturbation of the electron transport chain. Therefore bicarbonate signaling through sAC may provide a mechanism for metabolic feedback regulation during hypoxia. sAC differs from classical transmembrane adenylyl cyclase (tmAC) in its cellular localization and modulation, making it an excellent candidate for the regulation of physiological systems not under the control of classic hormone signaling. For example, carbon dioxide generation during metabolic processes or pH changes would alter intracellular bicarbonate concentration, thereby modulating sAC activity. sAC is present in virtually all tissues examined and its bicarbonate sensitivity has apparently been conserved between mammals and cyanobacteria (Zippin 2003).

1.10.3 Signaling Through Reactive Oxygen Intermediates

Mitochondria and their role as oxygen sensors within the cell have gained considerable attention within the field of hypoxic signaling. Reactive oxygen species (ROS), such as superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), are byproducts of respiration and generally considered toxic to cells. However, experimental evidence in mammalian cells suggests that ROS may participate in cell signal transduction pathways (Thannickal 2000).

Hypoxia partially inhibits mitochondrial electron transport, resulting in changes in the oxidation-reduction potentials in the electron carriers that increase the generation of ROS. Superoxide anion within the mitochondria is degraded by conversion to H_2O_2 by superoxide dismutase. The oxidants then enter the cytosol and function as second messengers. Many of the oxidants are degraded by the glutathione oxidase system (Holbrook 2002). Several groups have demonstrated that the stress activated p38 MAPK is also activated by intracellular ROS. Further evidence for the role of mitochondria derived ROS in activation of p38 was provided by the observations that inhibitors of the electron transport chain complexes I and II attenuated p38 activation. In addition, activation of p38 occurred within an hour of induced hypoxia in cardiomyocytes. However, the exact mechanism by which ROS activates gene expression is not known (Nathan 2003), (Holbrook 2002).

1.11 CONCLUSION OF LITERATURE

The pioneering work of Rogers, Sommerville, Davey, and Petronijevic has provided invaluable insight into the kinetics of exsheathment of trichostrongylid nematodes and has elicited speculation of the potential mechanisms of initial parasite development within the host. Their work has provided considerable evidence for the functional role of a CA in the exsheathment process. However, a gene encoding a CA has not been isolated and reported from

a trichostrongylid nematode parasite to date. In addition, virtually nothing is known about the developmental regulation of trichostrongylid nematodes.

Although the soil dwelling nematode *C. elegans* has provided considerable information about gene regulation and development of nematodes, *C. elegans* is not a parasite and its usefulness as a model for parasitic nematode development may extend only as far as the free-living stages of parasitic nematodes (Geary 2001). Consequently, in research aimed at understanding the molecular basis of parasitism, such as that presented in this work, the isolation and analysis of genetic material from the parasite of interest is essential. However, there is a dearth of information on developmental gene expression of trichostrongylid nematodes. Consequently, experimental design and formulation of hypotheses are limited to assumptions of similarity to biological pathways discovered from the model nematode *C. elegans* and mammalian systems. Review of the current literature regarding the signaling potential of molecular byproducts of a putative CA within trichostrongylid nematodes while suspended in the hypoxic environment of the host rumen provides a foundation for the following hypothesis:

Trichostrongylid nematodes transition from the free-living to the parasitic stage via receipt of a stimulus from the host rumen environment that is transduced and sustained, in part, through the activity of a CA leading to exsheathment.

This hypothesis was tested through the design of a series of experiments aimed at establishing an *in vivo* model of exsheathment, identifying a CA gene during the exsheathment process and characterizing its role in the initiation of exsheathment.

The specific objectives of these studies were to:

1. Determine the *in vivo* kinetics of exsheathment of *O. ostertagi* within the rumen environment of its bovine host.

2. Isolate mRNA and DNA from *O. osteragi* L3 during multiple time-points following exposure to the rumen.
3. Isolate a gene encoding a carbonic anhydrase from *O. ostertagi* L3 mRNA and DNA recovered in objective 2.
4. Quantitate the expression of carbonic anhydrase mRNA during the time course of exsheathment within the rumen.
5. Characterize as much as possible, the genomic architecture of a recovered carbonic anhydrase gene.

1.12 REFERENCES

- Ahluwalia, J.H. and A.G. Charleston (1974). Exsheathment of infective larvae of *Cooperia curticei*. *New Zealand Veterinary Journal* **22**(12): 237-238.
- Anya, A.O. (1976) Physiological Aspects of Reproduction in Nematodes: 267-337.
- Barrett, J. (1976). Studies on the induction of permeability into *Ascaris lumbricoides* eggs. *Parasitology* **73**: 109-121.
- Bilton, R.L. and G.W. Booker (2003). The subtle side to hypoxia inducible factor (HIF-1 α) regulation. *European Journal of Biochemistry* **270**: 791-798.
- Bird, A.F. (1955). Importance of proteases as factors involved in the exsheathing mechanism of infective nematode larvae of sheep. *Science* **121**: 107.
- Bird, A.F. and J. Bird (1998). Introduction to functional organization. *The Physiology and Biochemistry of Free-living and Plant-parasitic nematodes*. R. N. and W. D. J. Perry. New York, CABI Publishing: 1-20.
- Blaxter, M.L., A.P. Page, W. Rudin, and R.M. Maizels (1992). Nematode surface coats: actively evading immunity. *Parasitology Today* **8**: 243-247.
- Bonner, T.P. and M. Buratt (1976). The effect of actinomycin-D on development and infectivity of the nematode *Nippostrongylus braziliensis*. *International Journal for Parasitology* **6**: 289-294.
- Brady, H.J., N. Lowe, J.C. Sowden, M. Edwards and P.H. Butterworth (1991). The human carbonic anhydrase I gene has two promoters with different tissue specificities. *Biochemistry Journal* **277**(3): 903-905.

- Bruick, R.K. (2000). Expression of the gene encoding the proapoptotic Nip3 protein is induced by hypoxia. *Proceedings of the National Academy of Sciences* **97**: 9082-9087.
- Bunn, H.F. and R.O. Poyton (1996). Oxygen sensing and molecular adaptation to hypoxia. *Physiology Review* **76**: 839-885.
- Butterworth, P., J.H. Barlow, H.J.M. Brandy, M. Edwards, N. Lowe, and J.C. Sowden (1991). The structure and regulation of the human carbonic anhydrase I gene. *The Carbonic Anhydrases*. S. J. Dodgson, R.E. Tashian, G. Gros, and N.D. Carter. New York, Plenum Press.
- Cabiscol, E. and R. Levine (1996). The phosphatase activity of carbonic anhydrase III is reversibly regulated by glutathiolation. *Proceedings of the National Academy of Science* **93**: 4170-4174.
- Camenishch, G., D.M. Stroka, M. Gassmann and R.H. Wenger (2001). Attenuation of HIF-1 DNA-binding activity limits hypoxia-inducible endothelin-1 expression. *European Journal of Physiology* **443**: 240-249.
- Chegwidden, W. R. and N.D. Carter (2000). Introduction to the carbonic anhydrases. *The Carbonic Anhydrases*. S. J. Dodgson, R.E. Tashian, G. Gerolf, and N.D. Carter. Birkhauser, New Horizons.
- Chen, S.N. and R.E. Howells (1981). *Brugia pahangi*: uptake and incorporation of nucleic acid precursors by microfilariae and macrofilariae *in vitro*. *Experimental Parasitology* **51**: 296-306.
- Conrad, P.W., R.T. Rust, J. Han, D.E. Millihorn and D. Beitner-Johnson (1999). Selective activation of p38alpha and p38gamma by hypoxia. *Journal of Biological Chemistry* **274**: 33709-33713.
- Crofton, H.D. (1947). The second ecdysis of *Trichostrongylus retortaeformis*. *Parasitology* **38**: 101-103.
- Cypress R.H., E. A. Pratt and P. Van Zandt (1973). Rapid exsheathment of *Nematodspiroides dubius* infective larvae. *The Journal of Parasitology* **59**(2): 247-250.
- Davey, K.G. (1978). Molting in a parasitic nematode, *Phocanema decipiens*: the role of water uptake. *International Journal for Parasitology* **9**: 121-125.
- Davey, K.G., R.I. Sommerville and W.P. Rogers (1982). The effect of ethoxzolamide, an analogue of insect juvenile hormone, nor adrenaline and iodine on changes in the optical path difference in the excretory cells of oesophagus during exsheathment in *Haemonchus contortus*. *International Journal for Parasitology* **12**(6): 509-513.
- Davey, K.G. and S.P. Kan (1967). Endocrine basis for ecdysis in a parasitic nematode. *International Journal for Parasitology* **9**: 121-125.

- Davey, K.G. and W.P. Rogers (1982). Changes in water content and volume accompanying exsheathment of *Haemonchus contortus*. *International Journal for Parasitology* **12**(1): 93-96.
- Davey, K.G. and R.I. Sommerville. (1982). Changes in optical path difference in the oesophageal region and the excretory cells during exsheathment in *Haemonchus contortus*. *International Journal for Parasitology* **12**(6): 503-507.
- Dehority, B.A. (2003). *Rumen Microbiology*. Nottingham, University Press.
- Deitmer, J.W. and C.R. Rose (1996). pH regulation and proton signaling by glial cells. *Progresses in Neurobiology* **48**: 73-103.
- Deitmer, J.W. and H.P. Schneider (1998). Acid/base transport across the leech giant glial cell membrane at low external bicarbonate concentration. *Journal of Physiology* **512**(2): 459-469.
- Dell A. and H. R. Morris (2001). Novel Carbohydrate Structures. *Parasitic Nematodes Molecular Biology, Biochemistry and Immunology*. K. M. W. and H. W. Glasgow, CABI Publishing: 285-307.
- Desponsmmer D.D. and D.J. Jackson (1972). Actinomycin-D and puromycin-HCl in axenic culture of the nematode *Neoplectan glaseri*. *Journal of Parasitology* **58**: 774-777.
- Dmitri, C., T. Hofer, B. Christian, R.H. Wenger and M. Gassmann (2001). Hypoxia affects expression of circadian genes PER1 and CLOCK in mouse brain. *FASEB Journal* **264**: 2613-2621.
- Duranteau, J., N.S. Chandel, A. Kulisz, Z. Shao, and P.T. Schumacker (1998). Intracellular signaling by reactive oxygen species during hypoxia in cardiomyocytes. *Journal of Biological Chemistry* **273**(19): 11619-11624.
- Earnhardt, J.N., M. Qian, C. Tu, M.M. Lakkis, N.C. Bergenhem, P.J. Laipis, R.E. Tashian ND and D.N. Silverman (1998). The catalytic properties of murine carbonic anhydrase VII. *Biochemistry* **37**(30): 10837-10845.
- Epstein, A.C., J.M. Gleadle, L.A. McNeill, K.S. Hewitson, J. O'Rourke, D.R. Mole, M. Mukherji, E. Metzen, M.I. Wilson, A. Dhanda, Y.M. Tian, N. Masson, D.L. Hamilton, P. Jaakkola, R. Barstead, J. Hodgkin, P.H. Maxwell, C.W. Pugh, C.J. Schofield and P.J. Ratcliffe (2001). *C. elegans* EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. *Cell* **107**: 43-54.
- Fernley, R. T., R.D. Wright and J.P. Coghlan (1988). Complete amino acid sequence of ovine salivary carbonic anyhydrase. *Biochemistry* **27**: 2815-2820.

- Fujimoto, D. and S. Kanya (1973). Cuticulin: A noncollagen structural protein from *Ascaris* cuticle. *Archives of Biochemistry and Biophysics* **157**: 1-6.
- Ganesh, K. K., and J.B. Klein (2004). Analysis of expression and posttranslational modification of proteins during hypoxia. *Journal of Applied Physiology* **96**: 1178-1186.
- Geary, T.G., Blair, K.L., Ho, N.F.H., S.M. Sims and D.P. Thompson (1995). Biological functions of nematode surfaces. *Molecular Approaches to Parasitology*, J.C. and K. Boothroyd, (eds.). New York, Wiley-Liss: 191-205.
- Geary, T.G. and D.P. Thompson (2001). *Caenorhabditis elegans*: how good a model for veterinary parasites? *Veterinary Parasitology* **101**(3-4): 371-386.
- Gibbs, H.C. (1986). Hypobiosis in Parasitic Nematodes- An Update. *Advances in Parasitology* **25**: 129-173.
- Graven, K.K., L.H. Zimmerman, E.W. Dickson, G.L. Weinhouse and H.W. Farber (1993). Endothelial cell hypoxia associated proteins are cell and stress specific. *Journal of Cell Physiology* **157**: 544-554.
- Gu, Y. Z., J.B. Hogenesch, and C.A. Bradfield (2000). The PAS superfamily; sensors of environmental and developmental signals. *Annual Review of Pharmacology and Toxicology* **40**: 519-561.
- Heck, R.W., S.M. Tanhauser, R. Manda, C. Tu, P.J. Laipis and D.N. Silverman (1994). Catalytic properties of mouse carbonic anhydrase V. *Journal of Biological Chemistry* **269**: 24742-24746.
- Henry, R. P. (1984). The function of invertebrate carbonic anhydrases in ion transport. *Annals of the New York Academy of Science* **429**: 544-546.
- Hoffman, E.C., H. Reyes, F.F. Chu, F. Sander, L.H. Conley, B.A. Brooks and O. Hankinson (1991). Cloning of a factor required for activity of the Ah (dioxin) receptor. *Science* **252**: 954-958.
- Martindale, J.L. and N.J. Holbrook (2002). Cellular response to oxidative stress: signaling for suicide and survival. *Journal of Cellular Physiology* **192**: 1-15.
- Huang, L.E., J. Gu, M. Schau, and H.F. Bunn (1998). Regulation of hypoxia-inducible factor 1alpha is mediated by an O₂-dependent degradation domain via the ubiquitin-proteasome pathway. *Proceedings of the National Academies of Science* **95**: 7987-7992.
- Huaqi J., Guo, R. and A. Powell-Coffman (2001). The *Caenorhabditis elegans* hif-1 gene encodes bHLH-PAS protein that is required for adaptation to hypoxia. *Proceedings of the National Academy of Sciences* **98**(4): 7916-7921.

- Ivan, M., K. Kondo, H. Yang, W. Kim, J. Valiando, M. Ohh, A. Salic, J.M. Asara, W.S. Lane, and W.G. Kaelin Jr. (2001). HIF α targeted for VHL-mediated destruction by proline hydroxylation; implications for O₂ sensing. *Science* **292**: 464-468.
- Ivanov, S., S.Y. Liao, A. Ivanova, A. Danilkovitch-Miagkova, N. Tarasova, G. Weirich, M.J. Merrill, M.A. Proescholdt, E.H. Oldfield, J. Lee, J. Zavada, A. Waheed, W. Sly, M.I. Lerman and E.J. Stanbridge. (2001). Expression of hypoxia inducible cell-surface transmembrane carbonic anhydrases in human cancer. *American Journal of Pathology* **158**: 905-919.
- Harris, J.E. and H.D. Crofton (1957). *Journal of Experimental Biology* **34**: 116-130.
- Jaakkola, P., D.R. Mole, Y.M. Tian, M.I. Wilson, J. Gilbert, S.J. Gaskell, A. von Kriegsheim, H.F., Hebestreit, M. Mukherji, C.J. Schofield, P.H. Maxwell, C.W., Pugh, and P.J. Ratcliffe (2001). Targeting of HIF- α to the von Hippel-Lindau ubiquitylation complex by O₂ regulated prolyl hydroxylation. *Science* **292**: 468-472.
- Johnstone, I.L., Y. Shafi, A. Majeed and J.D. Barry (1996). Cuticular collagen genes from the parasitic nematode *Ostertagia circumcincta*. *Molecular and Biochemical Parasitology* **80**: 103-112.
- Khalifah, R.G. and D.N. Silverman. (1991). Carbonic anhydrase kinetics and molecular function. *The Carbonic Anhydrases: Cellular Physiology and Molecular Genetics*. S. J. Dodgson, R.E. Tashian, G. Gros, and N.D. Carter. New York, Plenum Press.
- Kita, K., H. Hirawake, and S. Takamiya (1997). Cytochromes in the respiratory chain of helminth mitochondria. *International Journal for Parasitology* **27**(6): 617-630.
- Kivela, A. J., J. Saarnio, T.J. Karttunen, J. Kivela, A.K. Parkkila, S. Pastorekova, J. Pastorek, A. Waheed, W.S. Sly, T.S. Parkkila and H. Rajaniemi (2001). Differential expression of cytoplasmic carbonic anhydrases, CAI and II, and membrane associated isozymes, CAIX and XII, in normal mucosa of large intestine and in colorectal tumors. *Digestive Disease Science* **46**(10): 2179-2186.
- Kivela, J., S. Parkkila, S. Metteri, A.K. Parkkila, A. Toivanen, and H. Rajaniemi (1997). Salivary carbonic anhydrase VI concentration and its relation to basic characteristics of saliva in young men. *Physiologica Scandinavica* **161**: 221-225.
- Kramer, J.M. (1997). Extracellular Matrix. *C. elegans* II. D. L. Riddle, Blumenthal, T., Meyer, B.J., Priess, J.R. Danvers, MA, Cold Spring Harbor Laboratory Press: 471-500.
- Krewnowska, K.D., M.R. Craig, S.R. Digiovanni, J.M. Mccarty, A.F.M. Moorman, W.H. Lamers, and A.C. Schoolwerth (2002). PEPCK mRNA localization in proximal tubule and gene regulation during metabolic acidosis. *Journal of Physiology and Pharmacology* **53**(1): 3-20.

- Lando, D., D.J. Peet, D.A. Whelan, J.J. Gorman and M.L. Whitelaw (2002). Asparagine hydroxylation of the HIF transactivation domain: a hypoxic switch. *Science* **295**: 858-861.
- LaPage, G. (1935). The second ecdysis of infective nematode larvae. *Parasitology* **27**: 186-206.
- Lee, D.L. (1965). The structure and composition of the helminth cuticle.: 187-254.
- Lewert, R.M. and C.L. Lee (1956). Quantitative studies of the collagenase-like enzymes of cercariae of *Schistosoma mansoni* and the larvae of *Strongyloides ratti*. *Journal of Infectious Diseases* **99**: 1-14.
- Lindskog, S. (1960). Purification and properties of bovine erythrocyte carbonic anhydrase. *Biochimica Biophysica Acta* **39**: 218-226.
- Lindskog, S. (1997). Structure and mechanism of carbonic anhydrase. *Pharmacological Theory* **71**: 1-20.
- Loncaster, J.A., A.L. Harris, S.E. Logue, J.P. Hunter, R.D. Wycoff, C.C. Pastoreck, J. Ratcliffe, P.J. Stanford and C.M.L. West. (2001). Carbonic anhydrase (CAIX) expression, a potential new intrinsic marker of hypoxia: correlations with tumor oxygen measurements and prognosis in locally advanced carcinoma of the cervix. *Cancer Research* **61**: 6394-6399.
- Lote, C.J. and J.B. Weiss (1971). Identification of digalactosylcysteine in a glycopeptide isolated from urine by a new preparative technique. *FEBS Letters* **16**: 81-85.
- Meldrum, N.U. and F.J.W. Roughton. (1933). Some properties of carbonic anhydrase, the carbon dioxide enzyme present in blood. *Journal of Physiology* **75**: 15-16.
- Minet, E., G. Michel, D. Mottet, M. Raes, and C. Michiels (2001). Transduction pathways involved in hypoxia-induced factor-1 phosphorylation and activation. *Free Radical Biology and Medicine* **31**(7): 847-855.
- Minyoung, L., J.T. Hwang, H.J. Lee, S.N. Jung, I. Kang, S.G. Chi, S.S. Kim and J. Ha (2003). AMP-activated protein kinase activity is critical for hypoxia inducible factor-1 transcriptional activity and its target gene expression under hypoxic conditions in DU145 cells. *Journal of Biological Chemistry* **278**(41): 39653-39661.
- Nagao, Y., J.S. Platero, A. Waheed and W.S. Sly (1993). Human mitochondrial carbonic anhydrase: cDNA cloning, expression, subcellular localization, and mapping to chromosome 16. *Proceedings of the National Academy of Sciences* **90**: 7623-7627.
- Nathan, C. (2003). Specificity of a third kind: reactive oxygen and nitrogen intermediates in cell signaling. *The Journal of Clinical Investigation* **111**(6): 769-778.

- Nishita, T., K. Matasuura, N. Ichihara, and M. Asari (2002). Isolation and measurement of carbonic anhydrase isoenzyme III in plasma, sera, and tissues of dogs. *American Journal of Veterinary Research* **63**(2): 229-235.
- Ozerol, N.H. and P.H. Silverman. (1972). Exsheathment phenomenon in the infective-stage larvae of *Haemonchus contortus*. *Journal of Parasitology* **58**(1): 34-44.
- Page, A.P. (2001). The Nematode Cuticle: Synthesis, Modification and Mutants. *Parasitic Nematodes Molecular Biology, Biochemistry and Immunology*. M. W. and H. Kennedy, W. New York, CABI Publishing: 167-193.
- Parkkila, S., K. Kaunisto, L. Rajaniemi, T. Kumpulainen, K. Jokinen, and H. Rajaniemi (1990). Immunohistochemical localization of carbonic anhydrase isoenzymes VI, II, and I in human parotid and submandibular glands. *Journal of Histochemical Cytochemistry* **38**: 941-947.
- Parkkila, S., A.K. Parkkila, T. Juvonen, A. Waheed, W.S. Sly, J. Saarnio, K. Kaunisto, S. Kellokumpu and H. Rajaniemi. (1996). Membrane-bound carbonic anhydrase IV is expressed in the luminal plasma membrane of the human gallbladder epithelium. *Hepatology* **24**: 1104-1108.
- Parkkila, S., A.K. Parkkila, J. Lehtola, A. Reinila, H.J. Sodervik, M. Rannisto and H. Rajaniemi (1997). Salivary carbonic anhydrase protects gastroesophageal mucosa from acid injury. *Digestive Disease Science* **42**: 1013-1019.
- Pax, R.A., T.G. Geary, J.L. Bennett and D.P. Thompson (1995). *Ascaris suum*: Characterization of transmural and hypodermal potentials. *Experimental Parasitology* **80**: 85-97.
- Petronijevic, T. and W.P. Rogers (1986). Organic and inorganic acids as a stimulus for exsheathment of infective juveniles of nematodes. *International Journal for Parasitology* **16**(2): 163-168.
- Petronijevic T., W.P. Rogers and R.I. Sommerville (1985). Carbonic acid as the host signal for the development of parasitic stages of nematodes. *International Journal for Parasitology* **15**(6): 661-667.
- Petronijevic, T. and W.P. Rogers. (1983). Gene activity and the development of early parasitic stages of nematodes. *International Journal for Parasitology* **13**(2): 197-199.
- Riddle, D.L. and A.S. Patrice (1997). Genetic and Environmental Regulation of Dauer Larva Development. *C. elegans II*. D. L. Riddle, T. Blumenthal, B.J. Meyer, J.R. Priess. Plainview, Cold Spring Harbor Press.
- Roberts, L.S. and G.D. Schmidt (2000). *Foundations in Parasitology*. Gerald D. Schmidt and Larry S. Roberts. (Ed) Foundations in Parasitology. St. Louis, McGraw Hill.

- Rogers, W.P. (1958). Physiology of the hatching of eggs of *Ascaris lumbricoides*. *Nature London* **181**: 1410-1411.
- Rogers, W.P. (1960). The physiology of infective processes of nematode parasite; the stimulus from the animal host. *Proceedings of the Royal Society of London. Series B. Biological Sciences* **152**(948).
- Rogers, W.P. (1962). *The Nature of Parasitism*. New York and London, Academic Press.
- Rogers, W.P. (1965). The role of leucine aminopeptidase in the moulting of nematode parasites. *Comparative Biochemistry and Physiology* **14**: 311-321.
- Rogers, W.P. (1980). The action of insect juvenile hormone on the hatching of eggs of the nematode, *Haemonchus contortus*, and its role in the development of infective and non-infective stages. *Comparative Biochemistry and Physiology* **66A**: 631-636.
- Rogers, W.P. (1982). Enzymes in the exsheathing fluid of nematodes and their biological significance. *International Journal for Parasitology* **12**(6): 495-502.
- Rogers, W.P. and F. Brooks. (1976). Zinc as a co-factor for an enzyme involved in exsheathment of *Haemonchus contortus*. *International Journal for Parasitology* **6**: 315-319.
- Rogers, W.P. and F. Brooks. (1977). The mechanism of hatching eggs of *Haemonchus contortus*. *International Journal for Parasitology* **6**: 315-319.
- Rogers, W.P. and R. I. Sommerville (1960). The physiology of the second ecdysis of parasitic nematodes. *Parasitology* **50**: 329-348.
- Rosen, O., C. Suarez, V.L. Schuster and L.P. Brion (2001). Expression of carbonic anhydrase IV in mouse placenta. *American Journal of Regulatory, Integrative, and Comparative Physiology* **280**(2): 365-375.
- Ruusuvuori, E., H. Li, K. Huttu, J.M. Palva, S. Smirnov, C. Rivera, K. Kaila and J. Voipio (2004). Carbonic anhydrase VII acts as a molecular switch in the development of synchronous gamma-frequency firing of hippocampal CA1 pyramidal cells. *Journal of Neuroscience* **24**(11): 2699-26707.
- Sanllorenti, P. M., J. Rosenfeld, V.P. Ronchi, P. Ferrara and R.D. Conde (2001). Two dimensional non-equilibrium pH gel electrophoresis mapping of cytosolic protein changes caused by dietary protein depletion in mouse liver. *Molecular and Cellular Biochemistry* **220**(1-2): 49-56.
- Semenza, G.L. and G.L. Wang. (1992). A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. *Molecular Cell Biology* **12**: 5447-5454.

- Silverman, P.H. and K.R. Podger. (1964). *In vitro* exsheathment of some nematode larvae. *Experimental Parasitology* **15**: 314-324.
- Slocombe, J.O.D. (1974). Further analyses of supernatant fluids from exsheathing infective *Haemonchus contortus* cayugensis larvae. *Journal of Parasitology* **57**: 801-807.
- Slocombe, J.O.D. and J.H. Whitlock. (1969). Rapid ecdysis of infective *Haemonchus contortus* cayugensis larvae. *Journal of Parasitology* **55**: 1102-1103.
- Smales, L.R. and R.I. Sommerville. (1976). Exsheathment of the infective larva of *Labiostrongylus eugenii*, a nematode parasite of the Kangaroo Island wallaby *Macropus eugenii*. *International Journal for Parasitology* **7**(3): 205-209.
- Sommerville, R.I. (1954). The second ecdysis of infective nematode larvae. *Nature*(174): 751.
- Sommerville, R.I. (1957). The exsheathing mechanism of nematode infective larvae. *Experimental Parasitology* **6**: 18-30.
- Sommerville, R.I. and M.A. Bailey (1973). *Nematospiroides dubius*: exsheathment of infective juveniles. *Experimental Parasitology* **33**(1): 1-9.
- Sterling, D., A.F. Reithmeier and J.R. Casey (2001). Carbonic Anhydrase: In the driver's seat for bicarbonate transport. *Journal of Pancreas* **2**(4): 165-170.
- Taylor, A. and J.H. Whitlock (1960). The exsheathing stimulus for infective larvae of *Haemonchus contortus*. *Cornell Veterinarian* **50**: 339-344.
- Thannickal, V.J. and B.L. Fanburg (2000). Reactive oxygen species in cell signaling. *American Journal of Physiological Lung and Cellular Molecular Physiology* **279**: 1005-1028.
- Tripp, B.C., S. Kerry, and J.G. Ferry (2001). Carbonic Anhydrase: New insights for an ancient enzyme. *The Journal of Biological Chemistry* **276**(52): 48615-48618.
- Tureci, O., U. Sahin, E. Vollmar, S. Siemer, E. Gottert, G. Seitz, A.K. Parkkila, G.N. Shah, J.H. Grubb, M. Pfreundschuh and W.S. Sly (1998). Human carbonic anhydrase XII: cDNA cloning, expression, and chromosomal localization of a carbonic anhydrase gene that is overexpressed in some renal cancers. *Proceedings of the National Academy of Science* **95**: 7608-7613.
- Wang, G.L. and G.L. Semenza (1995). Purification and characterization of hypoxia-inducible factor 1. *Journal of Biological Chemistry* **270**: 1230-1237.
- Wenger, R. (2000). Mammalian oxygen sensing, signalling and gene regulation. *Journal of Experimental Biology* **203**: 1253-1263.
- Wenger, R.H. (2002). Cellular adaption to hypoxia: O₂-sensing protein hydroxylases, hypoxia-inducible transcription factors, and O₂-regulated gene expression. *FASEB* **16**: 1151-1162.

- Whitney, P.L. and T.V. Briggie. (1982). Membrane-associated carbonic anhydrase purified from bovine lung. *Journal of Biological Chemistry* **257**(20): 12056-12059.
- Williams, J.C. (1987). Epidemiologic patterns of nematodiasis in cattle. *Veterinary Clinics of North American Food Animal Practice* **2**: 235-245.
- Williams, J.C. (1987). Infection with *Ostertagia ostertagi* in Goats and Calves. *Veterinary Parasitology* **23**: 51-67.
- Williams, J.C. and F.R. Bilkovich (1971). Development and survival of infective larvae of the cattle nematode, *Ostertagia ostertagi*. *Journal of Parasitology* **57**: 327-338.
- Wistrand, P.J. (1981). The importance of carbonic anhydrase B and C for the unloading of CO₂ by the human erythrocyte. *Acta Physiologica Scandania* **113**: 417-426.
- Wykoff, C.C., N.J.P. Beasley, P.H. Watdon, K. J. Turner, J. Pastorek, A. Sibtain, G. D. Wilson, H. Turley, K.L. Talks, P.H. Maxwell, C.W. Pugh, P.J. Ratcliffe and A.L. Harris. (2000). Hypoxia-inducible Expression of Tumor-associated Carbonic Anhydrases. *Cancer Research* **60**: 7075-7083.
- Zhu, X.L. and W.S. Sly (1990). Carbonic anhydrase IV from human lung. Purification, characterization, and comparison with membrane carbonic anhydrase from human kidney. *Journal of Biological Chemistry* **265**: 8795-8801.
- Zippin, J.H., Y. Chen, P. Nahirney, M. Kamenetsky, M.S. Wuttke, D.A. Fischman, L.R. Levin, and J. Buck (2003). Compartmentalization of bicarbonate-sensitive adenylyl cyclase in distinct signaling microdomains. *Federation of the American Societies of Experimental Biologists* **17**: 82-84.

CHAPTER 2

IN VIVO EXSHEATHMENT KINETICS OF OSTERTAGIA OSTERTAGI

2.1 INTRODUCTION

Ostertagia ostertagi is considered the most economically important gastrointestinal (GI) nematode parasite of cattle in temperate climates. Like all trichostrongylid nematodes, *O. ostertagi* has a direct life cycle consisting of three free-living stages, first stage larvae (L₁), second stage larvae (L₂) and the third (infective) larval stage (L₃). Transition from the L₁ to the L₂ stage occurs in the fecal pat and requires a molting process whereby the L₁ cuticle is shed, exposing the newly formed cuticle of the L₂. L₂ larvae also undergo a molting process as they develop to the L₃ stage. However, the L₂ cuticle, although separated from the underlying L₃ cuticle, is retained by the L₃. The retained L₂ cuticle completely encapsulates the L₃ and prevents it from feeding. It also affords this stage a higher tolerance to desiccation and cold temperatures, enabling it to survive for long periods on pasture. The L₃ subsequently migrate from the fecal pat onto surrounding herbage to await ingestion by the bovine host. Casting of the L₂ cuticle, retained on the L₃ marks the transition from the free-living to the parasitic stage and is described as the process of exsheathment. For trichostrongylid nematodes that infect the abomasum of cattle, L₃ exsheathment is initiated as this life cycle stage passes through the rumen (Sommerville 1957).

The exsheathment process in trichostrongylid nematodes has been studied extensively at the biochemical level (Davey 1982) (Rogers 1960) (Sommerville 1957). The culmination of data from these efforts strongly suggests that the enzyme carbonic anhydrase (CA) acts to initiate the exsheathment process. Davey and Rogers (Davey 1982), for example, demonstrated that exsheathment of *Haemonchus contortus* L₃ could be prevented by incubating larvae in the CA inhibitor ethoxzolamide prior to exposure to exsheathment media or rumen contents. While the

mechanism that CA may use to initiate exsheathment is unknown, it is hypothesized that this enzyme acts as a CO₂ sensor within the organism (Petronijevic 1985). Petronijevic (1985) suggested that, rather than the concentration of the components, the rate of exchange within the system, $H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$, and the rate of exchange of these ions within the larva itself may be important factors in stimulating exsheathment. Other factors reported to effect the exsheathment rate of *H. contortus*, *Trichostrongylus axei*, and *Teladorsagia circumcincta* L₃ are pH, oxidation reduction potential, temperature, CO₂ levels, concentration of a reducing agent and salt concentration of the media. These factors are presumed to affect the efficiency of the CA activity (Rogers 1960).

Although the biochemical evidence for the role of CA in initiation of exsheathment is convincing, a gene encoding a CA has not been reported from a parasitic nematode. Due to the absence of a molecular basis for the role of CA in exsheathment, a research objective was designed to isolate and subsequently characterize the expression of a gene encoding a CA from *O. ostertagi* L₃ following exsheathment in the bovine rumen. Pursuit of this objective first required a method to expose *O. ostertagi* L₃ to the rumen environment, and subsequently recover them during the exsheathment process. Such a method would not only enable the recovery of *O. ostertagi* L₃ for isolation of RNA, but would also provide a means to document the kinetics of *O. ostertagi* exsheathment in the bovine rumen, a phenomenon not previously reported. Consequently, the objectives of this study were to develop a procedure to expose *O. ostertagi* larvae to the bovine rumen environment and recover them during the exsheathment process. Accomplishing this objective would provide a method for the second objective of determining the rate of initiation of exsheathment of *O. ostertagi* L₃ following exposure to the rumen. The third objective was to investigate the effect of altered pH on the kinetics of *O. ostertagi* exsheathment in the bovine rumen.

2.2 MATERIALS AND METHODS

2.2.1 Maintenance of *Ostertagia ostertagi* Isolate

The *O. ostertagi* isolate used in this study was graciously provided by Dr. Louis Gasbarre, from the USDA laboratory in Beltsville, Maryland. This isolate was subsequently propagated at the LSU Agricultural Experiment Station, Ben Hur Research Station in Baton Rouge, Louisiana, by passage through several Holstein donor calves to provide a constant source of *O. ostertagi* eggs for subsequent culture to the L₃ stage.

2.2.2 Culture Preparation, Recovery, and Enumeration of *Ostertagia ostertagi* L₃

Pooled collections of feces taken directly from the rectum of donor calves were mixed with an adequate amount of tap water to form a slurry. An appropriate amount of course grade horticultural vermiculite was subsequently mixed and kneaded with the slurry to provide sufficient aeration and moisture retention of the culture. The culture mix was placed in a plastic box and covered tightly with aluminum foil. A number of 3 mm size holes were made in the foil cover for ventilation and the cultures were stored at room temperature of the laboratory (25°C ± 5°C) for 14 days. At the end of the incubation period the cultures were placed in screen-covered wire meshed baskets (6cm x 30cm²) lined with two layers of #50 grade Purewipe® cheese cloth (American Fiber & Finishing, Inc., Albemarle, N.C.) and placed overnight in a Baermann funnel. Baermann funnels were filled with warm tap water to a level that completely submerged the basket and culture material. The following morning, larvae from the culture material were transferred to a 50ml conical tube by releasing the stopcock on the tubing connected to the base of the funnel. The turbidity of the larval suspension was removed by multiple sedimentations in tap water. Larger debris was removed from the larval suspension by allowing larvae to migrate overnight at room temperature (25°C ± 5°C) through a 40 µm nylon mesh filter (Product No. 146-502, Spectrum Laboratories, Inc., Rancho Dominguez, CA) in a Baermann funnel. The

number of larvae in each collection was determined by counting larvae in an aliquot from the recovered larval suspension. Depending on the density of larvae, aliquots ranged from 10 μ l to 50 μ l. An average count of ten aliquots from each suspension was used to calculate the number of L₃ per ml of suspension. The volume of the suspension was adjusted according to the number of L₃ required per ml. In all cases, larvae were concentrated to a density greater than, or equal to, 1,000 L₃ per ml of suspension.

2.2.3 Rumen Cannulation

A rumen fistula was surgically established on a 14 month old Holstein steer. The surgical procedure was carried out with the animal in a standing position and confined in a conventional large animal squeeze chute. The first and second lumbar and twelfth thoracic nerves on the left side of the animal were blocked using a proximal paravertebral nerve block, by injecting 15 ml of lidocaine around each nerve at three injection sites per nerve. Following a surgical prep, a 9.5 cm diameter piece of skin was surgically removed from the left side of the paralumbar fossa. The peritoneum was then exposed and incised after blunt dissection through muscle layers. The rumen wall was subsequently pulled through the peritoneal incision, sutured to the muscle layers and skin using a simple interrupted pattern with #2 suture material and excised forming a fistula into the rumen. A rumen cannula with a 10cm center diameter (Bar Diamond Inc., Parma, ID, Product No. 9C) was fitted into the fistula and sealed with a plastic removable stopper provided by the manufacturer (Figure 2.1). A 2 wk post-operative convalescent period was observed prior to handling the cannula.

2.2.4 Animal Rations and Determination of Rumen PH

The temperature and pH of the rumen were recorded at each time-point during exsheathment of L₃ with a portable hand-held temperature and pH meter fitted with an extended probe. Prior to recording rumen pH, the meter was calibrated to the expected normal

temperature range of the rumen (38°C - 40°C) (Church 1976) according to the manufacture's instructions. To establish a near alkaline pH in the rumen, the fistulated steer was maintained on



Figure 2.1. The cannulated steer used in the exsheathment studies.

a 4-acre coastal bermuda grass pasture for 8 wk prior to conducting the exsheathment studies. After completing the exsheathment curves while the steer was on a grass diet, the same steer was subsequently relocated to a concrete floored pen, with no access to pasture or herbage. The steer was slowly acclimated to a high carbohydrate diet consisting of; 71% (4,200gm) cracked corn and 29% (1,700gm) of a 14% protein all stock concentrate pellet. The acclimation period was carried out over a 4 wk period and the steer was then maintained on the diet for an additional 3 wk. During the maintenance period the daily rumen pH measurements taken between 0900 and 1000 hr ranged from 4.8 to 5.5.

2.2.5 Exposure of *Ostertagia ostertagi* L₃ to Rumen Content

To determine the rate at which *O. ostertagi* L₃ exsheathed after exposure to rumen contents, aliquots of 3,000 *O. ostertagi* L₃ were transferred to 5mm diameter X 12cm long

cellulose ester disposable dialysis bags (Spectrum, Laboratories, Rancho Dominguez, CA Product No. 135-526). The dialysis bags had a maximum capacity of 1ml. Bags were secured with a 10 mm diameter threaded cap and possessed a molecular weight cut off of 300,000 Daltons. Dialysis bags were subsequently placed in a 5 cm X 15 cm tube made of polyvinyl chloride (PVC) (Figure 2.2.). Each end of the PVC tube had prefabricated threaded ends fitted with an open screw-on cap. The ends of the tube were covered with 5 mm x 5 mm wire mesh held in place with the screw-on cap and sixteen evenly spaced 1.5 cm holes were drilled into the tube. The wire mesh ends and holes in the tube enabled the rumen content fluids to flow freely into the tube (Figure 2.2). To facilitate easy recovery of the tube from the rumen, a 1.5 m length of cotton string was tied to the tube and the free end was secured to the outside cap of the rumen cannula. L₃ were submerged in rumen fluid by placing the tube at the floor of the rumen. A maximum of 9 dialysis bags could be placed in the PVC tube enabling recovery of larvae at 9 time points post-exposure to the rumen environment. Two PVC tubes, each containing 9 dialysis bags of larvae were at times used simultaneously, enabling analysis of 18 post-exposure time-points from one pool of L₃. The tube was subsequently removed at various time points post-exposure to recover larvae from the dialysis bags.

2.2.6 Exsheathment of *O. ostertagi* L₃ in the Rumen

Aliquots of 1,000 *O. ostertagi* L₃ were prepared by transferring appropriate volumes of larval suspension to a 1.5 ml eppendorf tube (Dot Scientific, Inc., Burton, MI, Product No. RN1500-GTT). Each aliquot was washed 3 times by centrifuging at 4,000 rpm at 4°C for 2 min in sterile filtered room temperature double distilled deionized water.

2.2.7 Calculation of Percent Exsheathment

Procedures for calculating percent exsheathment were based on those of Sommerville (1957) with some modifications. Immediately following retrieval from the rumen, rumen

contents were removed from the outside of the dialysis bags by a gentle rinse with .01M PBS. Larvae were quickly transferred from the dialysis bag to a 1.5 ml eppendorf tube using a disposable pipette supplied with each bag. To prevent further exsheathment from occurring, 1 to 2 drops of Lugol's iodine were immediately added to the larval suspension and mixed by gentle finger tipping. A range of 100 to 200 L₃ (10% to 15%) was examined to determine whether exsheathment had occurred. Larvae were considered exsheathed if the characteristic refractile ring (LaPage 1935) had formed around the circumference of the cuticle near the anterior end of the larvae. After formation of the refractile ring, the fracture and loss of the cuticle are physical processes dependent upon the movement of the larva. Larvae were judged not exsheathed if the refractile ring was not visible (Sommerville 1957). The percentage of exsheathed L₃ was determined by dividing the number of exsheathed L₃ by the number of L₃ examined.

2.3 RESULTS

2.3.1 *In vivo* Exposure of *Osteragia ostertagi* to Rumen Contents

Exposure of *O. ostertagi* L₃ to rumen contents and subsequent recovery at various time-points *via* the rumen cannula proved effective. Suspensions of L₃ recovered from the dialysis bags following exposure to the rumen were grossly and microscopically free of rumen particulates or infiltrate. Overall the method provided a means to examine the exsheathment process of *O. ostertagi in vivo*. In addition, clean suspensions of larvae were easily obtained for future RNA and DNA extractions.

2.3.2 Rumen PH and Temperature

The average pH of the rumen during the time course of each exsheathment study while the steer was on a grass diet was 6.6 with a range of 6.1 to 6.8. While on the concentrate diet, the mean rumen pH was 5.3 with a range of 4.8 and 5.5. The temperature of the rumen contents

ranged from 38.2°C to 39.0°C throughout the study period regardless of diet and was considered normal (38°C - 40°C) for fed animals (Church 1977) (Clarke 1976).



Figure 2.2. The dialysis bags (.005cm X 12cm) and PVC tube (5cm X 15cm) used in performing exsheathment experiments in the cannulated steer.

2.3.3 *Ostertagia ostertagi* Exsheathment in the Rumen

All exsheathment studies began between 0900 and 1000. The exsheathment rates of *O. ostertagi* L₃ following exposure to grain-derived and grass-derived rumen contents are shown in Figures 2.3A and 2.3B. Each time point in Figures 2.3A and 2.3B represents the mean of three separate exsheathment studies conducted on three separate days, with the exception of time points 5 min, 15 min, 30 min, and 45 min (Figure 2.3B). Only one exsheathment study was conducted that included these time points. Vertical lines at each time point in each figure represent the standard deviation of the means of each exsheathment study.

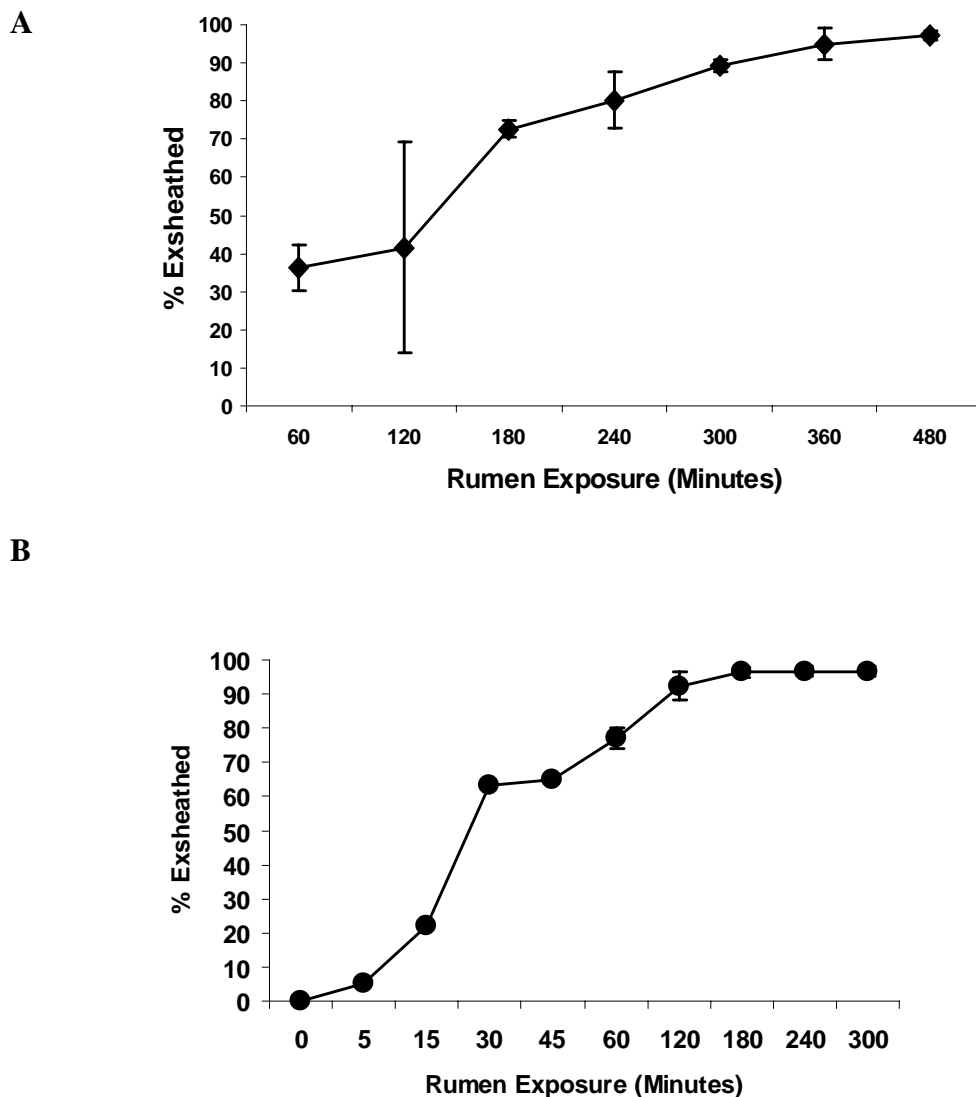


Figure 2.3. Percent exsheathment of *Ostertagia ostertagi* exposed *in vivo* to (A) grain-derived rumen contents or (B) grass-derived rumen contents. Each time point in Figures 2.3A and 2.3B represents the mean of three separate exsheathment studies conducted on three separate days, with the exception of time points 5 min, 15 min, 30 min, and 45 min (Figure 2.3B) where only one exsheathment study was conducted that included these time points. Vertical lines at each time point in each figure represent the standard deviation of the means of each exsheathment study.

Exsheathment of *O. ostertagi* L₃, when exposed to grain-derived rumen contents was two fold slower compared to larvae exposed to grass-derived rumen contents. Exsheathment reached a peak of 99% 360min post-exposure to grain-derived rumen contents (Figure 2.3A). In contrast,

the maximum exsheathment of L₃ exposed to grass-derived rumen contents was 98%, occurring at 180min following exposure to the rumen (Figure 2.3B).

2.4 DISCUSSION

The use of a rumen-fistulated bovine to expose and recover *O. ostertagi* larvae from the rumen provided an ideal method for determining exsheathment kinetics of *O. ostertagi*. The kinetics of *O. ostertagi* exsheathment observed in these experiments were similar to those observed in closely related sheep trichostrongylid nematodes (Rogers 1960). The maximum (98%) exsheathment rate of *O. ostertagi* occurred 180 min post exposure to grass-derived rumen contents (pH 6.6). These observations were similar to *in vitro* exsheathment profiles of *T. axei* L₃ in sheep. In 1960, Rogers and Sommerville observed that the maximum percentage of *T. axei* L₃ (93%) exsheathed 150 min post exposure to rumen contents of sheep at a pH of 6.5. In the same study, the maximum percentage of exsheathed *T. circumcincta* L₃ exposed to sheep rumen fluid adjusted to pH 6.5 occurred 150 min post exposure. These similar exsheathment profile data suggest that closely related nematodes might undergo exsheathment in the same manner and response to the same stimuli as *O. ostertagi*.

Further evidence for this can be found early in the exsheathment process. For example, the percentage of *O. ostertagi* L₃ that exsheathed following exposure to rumen contents for less than 15 min was negligible (5%). Although only one experiment was conducted at time points less than 60 min, these results are similar to observations reported by Rogers (1960) wherein which *T. axei* L₃ required an exposure time of greater than 12 min to initiate exsheathment and *H. contortus* required a 30 min exposure to rumen fluid before exsheathment would occur. This apparent delay in exsheathment initiation suggests these nematodes may need time to receive, and subsequently respond to a host factor before the exsheathment process can occur. A similar

hypothesis was proposed by earlier investigators (Petronijevic 1986; Petronijevic 1985), (Davey 1982), (Davey 1967) (Rogers 1960), (Sommerville 1957), (Sommerville 1954).

Interestingly, *O. ostertagi* L₃ exsheathed more rapidly when exposed to rumen contents while the steer was on a grass diet (pH 6.1 to 6.8) compared to exposure to grain-derived rumen contents (pH 4.8 to 5.5). One explanation for these differences in kinetics of exsheathment between the two diets may in fact be due to the different pH values, as well as other components of the rumen contents, resulting from each diet.

The pH of the rumen results from the amount of acid produced by the microflora present within it. It is mediated by the absorption and buffering capacity of the saliva that is produced by the animal (Dehority 2003) and can fluctuate considerably due to changes in diet. Indeed, pH is one of the most variable factors in the fermentation environment of the rumen (Hungate 1966). The impact of diet on rumen pH is largely mediated by the composition of roughage in the diet (Dehority 2003). The coarse-textured contents and bulk of roughage in the rumenoreticulum stimulates epithelial and tension receptors which then stimulate rumination. Rumination involves regurgitation, mastication and swallowing a bolus of previously ingested roughage. The increased frequency of chewing subsequently stimulates buccal mechanoreceptors, resulting in a significant increase in salivation. Studies have shown that cattle can produce from 75-150L of saliva per day (Hungate 1966; Church 1976).

The saliva of ruminants is alkaline (pH 8), having a high concentration of sodium and potassium bicarbonate and phosphate that buffers the acids produced by microorganisms in the rumen. Undissociated carbonic acid, bicarbonate and volatile fatty acids are the primary buffering components in the rumen between pH 5-7 (Counotte 1979). Additionally, saliva provides a nitrogen source for rumen microbes in the form of urea, mucin, phosphorus, magnesium and chlorine (Dehority 2003). Consequently, the increased salivation during

rumination results in the rumen pH being maintained at near neutral. This pH (pH 6.2-7.0) supports the growth and maintenance of cellulolytic bacteria whose metabolism also contributes to the maintenance of a neutral pH in the rumen by converting lactic acid, a strong acid, to a weaker propionic acid. It is of interest to note that a relatively high concentration of CO₂, bicarbonate, undissociated carbonic acid and a near neutral pH have been demonstrated to be essential components in initiating the exsheathment process *in vitro*. In addition, *in vitro* studies with *T. axei* and *H. contortus* L₃ have shown that a concomitant decrease in exsheathment rate occurs with decreased presence of bicarbonate (Rogers and Sommerville, 1960; Rogers, 1960). Lastly, it appears important to note that the biochemical conditions resulting in the rumen from a high roughage diet provide a high concentration of substrate for the activity of CA.

In contrast to a grass or high roughage rumen environment, grain-derived rumen contents should have a lower concentration of carbonic acid and bicarbonate and the rumen pH should, therefore, be lower. The reduction in these components is due primarily to a substantial decrease in rumination that accompanies a high grain or low roughage diet. The epithelial and tension receptors of the rumen are not activated to the level experienced while on a high roughage diet, due to the fine texture and low bulk content in the rumen associated with a high grain diet. In fact, during the current studies the rumen content was extremely fluid for the duration of the grain feeding time course. The reduced receptor activation leads to a significant reduction in rumination and concomitant reductions in chewing and salivation. Thus, the buffering component (saliva) of the rumen is diminished, which also leads to a lower rumen pH.

Also associated with the reduced rumen motility is a decrease in mixing and absorption, so that the potential for volatile fatty acid:bicarbonate exchanges across the rumenoreticulum epithelium is reduced at a time when increased amounts of bicarbonate would be advantageous to combat rising acidity (Leek 1993). Growth of cellulolytic bacterial species such as

Ruminococcus spp., *Butyrivibrio spp.* and *Eubacterium spp.* begin to decrease at pH 6.5 and are virtually eliminated at pH 5.9 (Russell 1980). The cellulolytic bacteria are replaced with the more acid tolerant amylolytic bacteria that are important for starch fermentation. The amylolytic bacteria, however, produce substantial amounts of lactic acid that further add to a reduction in the rumen pH. In this study, when rumen pH measurements were between 4.8 and 5.5, it was presumed that the predominant bacterial populations were amylolytic bacteria and thus a high lactic acid concentration likely existed during the grain-feeding portion of the exsheathment studies.

It seems likely, both from previous studies and the current work, that there is a strong link between the rate of *O. ostertagi* exsheathment and the biochemistry of the rumen environment. If the exsheathment of *O. ostertagi* is initiated through the activity of a CA, as has been suggested through biochemical studies in other trichostongylids, it is axiomatic that this enzyme may be activated earlier, and at greater levels, in larvae exposed to a grass-derived rumen content (increased substrate concentrations of undissociated carbonic acid and bicarbonate), compared with grain-derived rumen content. The result of this increased activation, then, would be an increased rate of exsheathment, as observed with *O. ostertagi* incubated in grass-derived rumen contents. The future isolation and quantitation of CA expression, if it exists, should help clarify the role of this molecule during the exsheathment process.

The variation in the kinetics of exsheathment of *O. ostertagi* L₃ exposed to rumen contents derived from different rations should be considered when artificially infecting animals for research purposes, such as drug efficacy studies. It appears for *O. ostertagi* that exsheathment efficiency is greater when the rumen pH is near alkaline. It is not known, however, if larvae that experience a delay in exsheathment are less successful in establishing an infection within the predilection of the abomasum. It should be noted that during the time course of this

study, one experiment was conducted in which sheathed larvae were exposed to the rumen for 24 consecutive hours and then subsequently recovered (data not shown). Starting at 8hr post exposure, *O. ostertagi* larvae generally became lethargic in their motility. At 12hr greater than 50% of the larvae were dead and by 24hr 100% of the larvae recovered, although exsheathed, had died. These data suggested that *O. ostertagi* L₃ must exit the rumen within 12hr following exsheathment. This apparent limited tolerance for the rumen environment may partially explain the relatively low establishment of artificial infections of *O. ostertagi* and other trichostrongylids experienced under laboratory conditions, as well as under natural conditions. A 24hr exposure, however, was not conducted while the steer was on a grass diet, and thus, *O. ostertagi* may have a longer longevity in the more alkaline grass-derived rumen contents. Before conclusions can be drawn regarding the longevity of *O. ostertagi* in the rumen, a more systematic approach needs to be applied to this question.

In conclusion, the use of disposable dialysis bags as a vehicle to expose *O. ostertagi* L₃ to the rumen environment for subsequent recovery proved useful for determining the kinetics of exsheathment. This method should also prove useful for gene expression studies aimed at identifying genes required for establishment of infection. This method will be used in experiments aimed at identifying a CA gene(s) putatively responsible for initiating the exsheathment process in trichostrongylid nematodes.

2.5 REFERENCES

- Church, D.C. (1976). *Digestive Physiology and Nutrition of Ruminants*. Corvallis, O.S.U Book Stores, Inc.
- Clarke, R.T.J. (1977). The gut and its micro-organisms. *Microbial Ecology of the Gut*. R. T. J. Clarke and T. Bauchop. New York, Academic Press: 36-71.
- Counotte, G.H.M. (1979). An analysis of the buffer system in the rumen of dairy cattle. *Journal of Animal Science* **49**: 1536-1544.

- Davey, K.G., R.I. Sommerville and W.P. Rogers (1982). The effect of ethoxzolamide, an analogue of insect juvenile hormone, nor adrenaline and iodine on changes in the optical path difference in the excretory cells of oesophagus during exsheathment in *Haemonchus contortus*. *International Journal for Parasitology* **12**(6): 509-513.
- Davey, K.G. and S.P. Kan (1967). Endocrine basis for ecdysis in a parasitic nematode. *International Journal for Parasitology* **9**: 121-125.
- Davey, K.G. and W.P. Rogers (1982). Changes in water content and volume accompanying exsheathment of *Haemonchus contortus*. *International Journal for Parasitology* **12**(1): 93-96.
- Dehority, B.A. (2003). *Rumen Microbiology*. Nottingham, University Press. U.K.
- Hungate, R.E. (1966). *The Rumen and Its Microbes*. New York, NY, Academic Press.
- LaPage, G. (1935). The second ecdysis of infective nematode larvae. *Parasitology* **27**: 186-206.
- Leek, B.F. (1993). Digestion in the Ruminant Stomach. *Duke's Physiology of Domestic Animals*. M.J.A.R. Swenson, W.O. Ithaca, Cornell Press.
- Petronijevic, T., and W.P. Rogers (1986). Organic and inorganic acids as a stimulus for exsheathment of infective juveniles of nematodes. *International Journal for Parasitology* **16**(2): 163-168.
- Petronijevic T., W.P. Rogers and R.I. Sommerville (1985). Carbonic acid as the host signal for the development of parasitic stages of nematodes. *International Journal for Parasitology* **15**(6): 661-667.
- Rogers, W.P. and R.I. Sommerville. (1960). The physiology of the second ecdysis of parasitic nematodes. *Parasitology* **50**: 329-348.
- Russell, J.B. and D.B. Dombrowski (1980). Effect of pH on the efficiency of growth by pure cultures of rumen bacteria in continuous culture. *Environmental Microbiology* **39**: 604-610.
- Sommerville, R.I. (1954). The second ecdysis of infective nematode larvae. *Nature* **174**: 751.
- Sommerville, R.I. (1957). The exsheathing mechanism of nematode infective larvae. *Experimental Parasitology* **6**: 18-30.

CHAPTER 3

ISOLATION AND QUANTITATIVE MEASUREMENT OF GENE EXPRESSION OF CARBONIC ANHYDRASE FROM *OSTERTAGIA OSTERTAGI* DURING *IN VIVO* EXSHEATHMENT OF INFECTIVE LARVAE

3.1 INTRODUCTION

The first event in the infection process of trichostrongylid nematodes of ruminants is exsheathment. Exsheathment occurs in the rumen for trichostrongylid species that infect the abomasum of cattle and involves the casting of the second larval stage (L₂) cuticle retained by the infective third-stage larvae (L₃). The exsheathment process presumably is initiated through the activity of the enzyme carbonic anhydrase (CA) (Petronijevic 1985). The effector mechanism, however, resulting from the activity of this enzyme leading to exsheathment is not known. It is hypothesized that the turnover rate of one or more of the components within the chemical reaction mediated by CA ($\text{CO}_2 + \text{H}_2\text{O} \leftrightarrow \text{H}_2\text{CO}_3 \leftrightarrow \text{HCO}_3^- + \text{H}^+$) is responsible for initiating exsheathment. One theory suggests that a sudden decline in intracellular pH leads to a signaling cascade resulting in the breakdown of excretory cell membranes and the subsequent release of exsheathment fluid. It is also thought that cellular development and differentiation to the fourth larval stage (L₄) is initiated within this putative signaling event, originating with CA activity (Davey 1982a; Davey 1982b; Petronijevic 1986).

The theoretical link between CA activity and initiation of gene transcription leading to development to the L₄ stage is based on an *in vitro* study with *Haemonchus contortus* (Petronijevic 1983). Sheathed *H. contortus* L₃ were exposed to $8 \times 10^{-7}\text{M}$ and $8 \times 10^{-6}\text{M}$ of the DNA transcription inhibitor actinomycin-D, and stimulated with 42% CO₂ at 39°C to initiate exsheathment. Following exposure to stimulating conditions for exsheathment, L₃ were transferred to media and conditions conducive for development to L₄, the first parasitic stage. A similar percentage of larvae exposed to both concentrations of actinomycin-D exsheathed,

compared to non-treated larvae. However, only 7% of the L₃ exposed to 8×10^{-6} M actinomycin-D developed to the L₄, while greater than 60% of untreated L₃ and L₃ exposed to 8×10^{-7} M actinomycin-D subsequently developed to the L₄ (Petronijevic 1983). Similarly, exposure of *Nippostrongylus braziliensis* L₃ (Bonner 1976), a nematode parasite of rodents, and *Neoaplectana glaseri* (Desponsmmer and Jackson 1972) a nematode parasite of insects, to 8×10^{-6} M and 2.5×10^{-5} M actinomycin-D, respectively, prevented further development of these parasites to the first parasitic stage. However, exsheathment was apparently unaffected by exposure to these concentrations of actinomycin-D, as both species readily exsheathed both *in vitro* and *in vivo*. These observations suggest that the genes governing exsheathment, such as genes for CA, are transcribed during the L₂ stage, but the genes required for development to the first parasitic stage are controlled at the transcriptional level in the L₃ stage following initiation of exsheathment (Petronijevic 1983).

The biochemical evidence supporting the role for the activity of a CA in initiating exsheathment and potentially serving as a biological “trigger” for development to the first parasitic stage is convincing. Partial support for the role of this enzyme comes from the reported failure of *H. contortus* L₃ to exsheath following exposure to the CA inhibitor ethoxzolamide (Davey 1982). Additional evidence comes from the culmination of data obtained from biochemical studies showing varied substrate concentrations for CA result in alterations in exsheathment efficiency of trichostrongylid nematodes *H. contortus*, *Trichostrongylus axei*, and *Teladorsagia circumcincta* (Petronijevic 1986), (Petronijevic 1985), (Rogers 1962), (Rogers 1960a), (Rogers 1960b), (Rogers 1958), (Sommerville 1957), (Sommerville 1954).

With the support of the reported roles of CAs described in other biological systems, it is not unreasonable to suggest that trichostrongylid nematodes may utilize a byproduct of CA

activity as an early signaling molecule for development to the first parasitic stage. A CA gene, however, has not been isolated and reported from a parasitic nematode.

The objectives of this study were to isolate a CA gene from *O. ostertagi* and to quantitatively measure its expression during *in vivo* exsheathment in the rumen.

3.2 MATERIALS AND METHODS

3.2.1 Isolation of RNA from *Ostertagia ostertagi*

L₃ with their retained second stage sheath were isolated from fecal cultures obtained from donor calves, as previously described in Chapter 1, Section 2.2. The larvae were washed once in PBS by centrifugation at 5,000 rpm for 1 min, then snap frozen as a dry pellet in liquid nitrogen. Exsheathed larvae were produced using the *in vivo* model described previously (Chapter 1, 2.2). Briefly, larvae were incubated in the rumen for various time-points. Following removal from the rumen, L₃ were washed in PBS by centrifugation and snap frozen in liquid nitrogen as a dry pellet. L₃ were stored at -70°C until required.

Pellets of frozen L₃ were transferred to a mortar and pestle and ground into a fine powder under liquid nitrogen. This powder was resuspended in 0.5 ml of RNeasy 60 (Tel-test, Friendship, TX) and total RNA was isolated as per the manufacturer's directions (Tel-test). Briefly, 100 µl of chloroform was added to the 0.5 ml RNeasy sample, the sample was mixed and centrifuged at 12,000 rpm for 10 minutes at 4°C. Following centrifugation the top, aqueous phase containing the RNA was removed to a clean tube and the RNA was precipitated with 0.8 volumes of 100 % isopropanol overnight at -20°C. The RNA was then pelleted by centrifugation at 12,000 rpm for 25 min, at 4°C. The pellet was washed with 1 ml of 75% cold ethanol, air dried at room temperature for 10 minutes and resuspended in 80 µl Diethylpyrocarbonate (DEPC) treated water by heating at 65°C for 10 min. RNA concentrations were calculated using the

Warburg-Christian assay generated by an automated spectrophotometer. Integrity of the RNA was determined by agarose gel analysis.

3.2.2 Reverse Transcription of cDNA

3.2.2.1 Oligo-d(T) PCR Anchor Primer

Reverse transcription was carried out on 1200 ng RNA using Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT). Thirty-five μ l containing 1200 ng RNA was denatured at 65°C for 10 minutes and placed immediately on ice for 2 minutes. Forty-five μ l of master mix was subsequently added to the RNA. Master mix consisted of 16 μ l 5x first strand buffer (Invitrogen, Carlsbad, CA), 4 μ l 0.1M DTT (Invitrogen), 2 μ l 10mM dNTP (Invitrogen), 4 μ l 150 μ M oligo d(T) (Genelab, Louisiana State University, Baton Rouge, LA), 3 μ l 1 X bovine serum albumin (BSA) (New England Biolabs, MA), 1.5 μ l RNAsin (Invitrogen), 1.5 μ l double distilled water and 3 μ L M-MLV RT (Invitrogen). Reactions were incubated at room temperature for 10 minutes to allow the oligo d(T) primer to anneal to the denatured RNA, then at 40°C for one hr for reverse transcription to occur. Following the hour incubation, the cDNA was placed on ice for 2-5 min to cool, pulsed at 12,000 rpm to bring the contents to the bottom of the tube, and stored at -20°C until required.

3.2.2.2 SuperSMART™ CDNA Synthesis

Synthesis of cDNA was also carried out according to the superSMART™ cDNA synthesis protocol (Clontech, BD Biosciences, Palo Alto, CA). This protocol utilizes an oligo(dT) primer (3' SMART™ CDS Primer II A) to prime the first-strand synthesis reaction. When the reverse transcriptase reaches the 5' end of the mRNA, the enzyme's terminal transferase activity adds additional deoxycytidine residues to the 3' end of the cDNA. The SMART™ oligonucleotide has an oligo(G) sequence at its 3' end that base pairs with the

deoxycytidine stretch, creating an extended template. The reverse transcriptase then switches templates and continues replicating to the end of the oligonucleotide. The resulting full-length, single stranded cDNA contains the complete 5' end of the mRNA and sequences complementary to the SMART™ oligonucleotide. sSMART cDNA synthesis was carried out by adding 1.0 µg of exsheathed *O. ostertagi* L3 RNA to 7 µl of 3' SMART CDS Primer II A (12µM), 7 µl SMART II A oligonucleotide (12µM) and deionized H₂O until the volume of the reaction was 64 µl. This mixture was then incubated in a thermalcycler for 2 min at 65°C to denature the RNA. The temperature was subsequently reduced to 42°C. First strand synthesis was then carried out by adding 20 µl 5X first strand synthesis buffer, 2 µl dithiothreitol (10mM), 10 µl 50X dNTP (10mM), 5 µl RNase inhibitor (20U/µl) and 5 µl PowerScript Reverse Transcriptase to the 64 µl RNA sample. The entire reaction (106 µl) was subsequently incubated at 42°C for 30 min in the thermalcycler to allow the Reverse Transcriptase to produce 1st strand cDNA. The reverse transcription reaction was terminated at the end of the 30 min incubation by the addition of 2 µl of EDTA.

Unincorporated nucleotides and small cDNA fragments (< 100 bases) were removed from the 1st strand cDNA reaction by column chromatography using the NucleoSpin® Extraction procedure (Clontech BD Biosciences, Palo Alto, CA). This procedure purifies cDNA by a size exclusion matrix using centrifugation to force small products through the matrix. In this procedure the cDNA was mixed with 3 times its volume of NT2 buffer and loaded onto a NucleoSpin Extraction Spin column contained within a 2 ml collection tube. The tube with column was centrifuged for 1 min at 14,000 rpm and the resultant flow through and collection tube was then discarded. The extraction column was then transferred to a fresh collection tube and 500 µl of NT3 buffer was added to the column and centrifuged at 14,000 rpm for 1min. This step was repeated 3 times. The column filter matrix was then dried by centrifugation at 14,000

rpm for 1 min and transferred to a fresh collection tube. The cDNA was eluted from the column filter matrix by adding 50 µl of sterile distilled water, incubating at room temperature for 2 min, and centrifuging for 1 min at 14,000 rpm. An additional 35 µl of sterile distilled water was loaded onto the column, incubated and centrifuged as previously described, yielding a final volume of 85 µl of purified sSMART cDNA.

The purified single stranded sSMART cDNA was then PCR amplified to produce double stranded cDNA according to sSMART cDNA synthesis protocol supplied by the manufacturer (Clontech, BDBiosciences, Palo Alto, CA). Firstly, 2.5 µl of the purified sSMART cDNA was added to 77.5 µl of sterile distilled water. A PCR master mix was prepared by combining 4 µl deionized water, 10 µl 10X Advantage 2 PCR buffer, 2 µl 50X dNTP (10mM), 2µl 5' PCR primer II A (12 µM), and 2 µl 50X Advantage 2 polymerase mix. A PCR reaction was subsequently carried out by combining 20 µl of the PCR master mix with 80 µl of the diluted sSMART cDNA. The PCR conditions consisted of an initial denaturation of 95°C for 1 min followed by 23 cycles of 95°C for 5 sec, 65°C 5 sec, and 68°C for 3 min.

3.2.3 Carbonic Anhydrase Primer Design

Several CA-encoding cDNA's have been isolated from *Caenorhabditis elegans*. These gene sequences are available on the internet through both the National Center for Biotechnology (NCBI) (www.ncbi.nlm.nih.gov) and the *C. elegans* home page (Wormbase) (www.wormbase.org). In addition, a set of expressed sequence tags (ESTs) from *O. ostertagi* are available at www.nematode.net. The *C. elegans* gene sequences were used to search the *O. ostertagi* EST database, using the BLAST algorithm located at www.nemablast.com. Once an *O. ostertagi* EST was identified, primers were designed from that EST and used to PCR amplify a section of the CA transcript from the prepared exsheathed L3 cDNA (Figure 3.2). The primers used were designated OoCAF1 and OoCAR1 and are listed in Table 3.1.

3.2.4 Isolation of Carbonic Anhydrase Gene from Exsheathed L₃ CDNA

All polymerase chain reactions were conducted using a Primus 96 ^{plus} thermocycler. The primers OoCAF1 and OoCAR1 were used to amplify a putative CA gene from *O. ostertagi* cDNA, using Advantage 2 DNA Polymerase (Clontech) as per the manufacturers' directions. In brief, each 25 µl reaction contained 2.5 µl 10x Advantage 2 buffer, 0.5 µl Advantage 2 Taq Polymerase, 1 µl 10 mM dNTP, 19 µl of double distilled water and 0.5 µl exsheathed L₃ sSMART cDNA. Reaction conditions consisted of an initial denaturation at 95°C for 1 min., followed by 30 cycles of 95°C 15 sec, 70°C 20 sec and 68°C 1 min, with a 7 min extension at 68°C. In some instances, the resultant amplicon was used as template in a secondary PCR with the primer OoCAF2 as a nested sense primer and OoCAR1 as the antisense primer.

PCR products were analyzed by 1.2 % agarose gel electrophoresis. Agarose gels were prepared by adding 1.2 g agarose (Amresco, Solon, OH) to 100 ml 1x TAE (40mM Tris-acetate, 1mM EDTA). Ethidium bromide (2µg/ml) was added to the agarose to allow visualization of the DNA. Electrophoresis was carried out at 10 volts per cm of gel length, using BioRad Electrophoresis systems (BioRad, Hercules, CA).

3.2.5 Cloning of Carbonic Anhydrase from *Ostertagia ostertagi*

PCR products produced using OoCAF1 and OoCAR1 that matched the desired size in base pairs (bp) were excised from the agarose gel using a sterile scalpel blade. DNA products were isolated from the gel using the UltraClean™GelSpin procedure (MoBio Inc., Carlsbad, CA). This procedure purifies the DNA from the agarose gel using a spin filter format. The agarose slice containing the PCR product of interest was melted at 65°C in 300 µl of GelBind Buffer (NaClO₄ solution). This solution was then applied to a spin filter basket, which was centrifuged at 10,400 rpm for 10 sec. At this step, the agarose passes through the column while the DNA binds to the column matrix. The spin column basket was then removed from the tube

and the flow-through was mixed by vortexing for 5 sec. The flow-through was subsequently reloaded onto the spin filter and centrifuged for 10 sec at 10,400 rpm. Following centrifugation, the flow-through was discarded and 300 µl of Gel Wash buffer (Tris / Ethanol solution) was

Table 3.1. Listing of primers used in isolation of full-length cDNA encoding carbonic anhydrase from exsheathed third stage *Ostertagia ostertagi*.

PRIMER NAME	SEQUENCE	USE
OoCASTRT	5- atggcgtttctcagccaactctcag-3'	Full length CA Isolation
OoCAF1	5'-ctc gac tca ctt ggc aaa gta ctg cac g-3'	CA Isolation Forward Primer
OoCAF2	5'-gga gtt ggc ggt gtc gtt tct gcc a -3'	CA Isolation Nested Forward
OoCAR1	5'-gtc gtg tga ctc att gag aaa gat ggc gat c-3'	CA Isolation Reverse
OoCAR2	5'-gcg tat cca acc cct cct agt gtg tgt tc -3'	CA Isolation Nested Reverse
Nematode Splice Leader 1	5'-tgt aga att ccg cgg ttt aat tac cca agt ttg-3'	5' End Isolation
SMART II A Oligonucleotide	5-'aag cag tgg tat caa cgc aca gta gcg gg- 3'	SMART cDNA synthesis
Oligo-dT anchor	5'-gac cac gcg tat cga tgt cga ctt ttt ttt ttt ttt- 3'	3' End Isolation
3' CDS Primer IIA	5'-aag cag tgg tat caa cgc aga gta ct ₍₃₀₎ NN-3'	SMART cDNA synthesis and 3' Isolation
M13F	5'-gta aaa cga cgg cca-3'	Amplify cloned PCR products
M13R	5'- gga aac agc tat gac cat g-3'	Amplify cloned PCR products

loaded onto the basket and centrifuged for 10 sec at 10,400 rpm. This step removes potential residual agarose TAE and GelBinding buffer from the filter matrix. Following the last wash the column was centrifuged again to remove any last remnants of wash buffer, then transferred to a

clean microfuge tube. Fifty µl of double distilled water was added to the basket to elute the PCR product from the matrix. Following a 1 min incubation at room temperature, the column was centrifuged at 10,400 rpm. The water, containing the eluted PCR product was stored at -20°C.

PCR products of interest were cloned into the pTOPO®-(BluntII or TA) vector (Invitrogen) for sequencing and analysis. Cloning was as per the manufacturers' direction and comprised the mixing of 3 µl of purified PCR product with 1µl of pTOPO®-BluntII or pTOPO®-TA vector and 1µl of 1.2M NaCl. These reagents were incubated at room temperature for 10-30 minutes to allow insertion of the DNA fragment into the pTOPO®- vector. Two µl of the ligation reaction was then added to a 25 µl aliquot of One Shot-Top10® DH5α™ -T1® *Eschericia coli* chemically competent cells (Invitrogen), previously thawed on ice. The ligation and cells were incubated on ice for 30 min, heat shocked at 42°C for 30 seconds, then cooled on ice for 2 min. Two hundred and fifty µl of SOC media (2% tryptone, 0.5% yeast extract, 10mM NaCl, 2.5mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, 20mM glucose) was then applied to the cells and the entire tube was incubated at 37°C for 1 hr, with shaking. Following the incubation, 50 µl of the transformation was plated onto Luria Bertani (LB) (10 gm bacto-tryptone, 5 gm yeast extract, 10 gm NaCl, pH adjusted to 7.5 with NaOH, autoclave sterilized) plates containing 150 µg/ml kanamycin. The plates were incubated overnight at 37°C.

Following the overnight incubation, 5 colonies from each plate were selected and analyzed for the presence of an insert by PCR. A PCR master mix was prepared consisting of 2.5 µl 10x first strand buffer (25 mM MgCl₂, 1 µl 10 mM dNTP, 0.5 µl each of M13F and M13R primer (20 µM), 19 µl double distilled water and 0.5 µl AmpliTaq (Applied Biosystems, Foster City, CA). This master mix was dispensed into individual PCR tubes (24 µl per tube). A sterile pipette tip was then used to transfer a colony from a LB plate to the master mix. After the

colony was added to the PCR mix the tip was also used to either inoculate 2 ml of LB media containing kanamycin (100 µg/ml) or to “patch” the colony across to a fresh plate. In this way fresh stocks of each colony were grown up overnight. The M13F and M13R primers bind to regions of homology within the pTOPO® vector and amplify the region across the cloning site of the vector. The size of the PCR product, then, is informative as to whether a clone is recombinant or empty. The reaction conditions for the PCR colony check were an initial denaturation at 95°C for 1 min., followed by 25 cycles of 95°C 30 sec, 50°C 30 sec, 72° 1 min. PCR products (8 µl) were analyzed using agarose gel electrophoresis as described above.

3.2.6 Isolation of Carbonic Anhydrase 5' End by PCR

The cDNA clone isolated from exsheathed L3 cDNA encoded only the middle portion of the CA (CA), when compared to the known gene sequence of the *C. elegans* CA, *cah-6*. In order to isolate the 5' end of *O. ostertagi* CA, the nematode splice leader 1 (NSL1) primer was used for the sense primer and OoCAR1 was used as the antisense primer in a PCR reaction with exsheathed *O. ostertagi* L₃ as a template. A PCR master mix was prepared using the Advantage 2 polymerase as previously described except a 50 µl reaction was used in this case. The PCR conditions of the reaction consisted of an initial 30 sec denaturation at 95°C followed by 25 cycles of 94°C for 30 sec, 66°C for 20 sec, 68°C for 2 min and a 7 min extension at 68°C was carried out at the end. Reaction products were analyzed by agarose gel electrophoresis and cloned as described above.

3.2.7 Isolation of 3' Carbonic Anhydrase CDNA Sequence by PCR

The 3' end of the cDNA encoding the *O. ostertagi* CA was isolated from exsheathed L3 cDNA produced with the sSMART cDNA synthesis protocol (Clontech BD Biosciences, Palo Alto, CA). Briefly, 0.5 µl of exsheathed *O. ostertagi* L₃ cDNA produced using the sSMART cDNA synthesis protocol was used as template in a PCR reaction in order to obtain the 3' prime

end of the CA gene. A PCR master mix was prepared that consisted of 2.5 µl 10X Advantage 2 PCR buffer, 1.0 µl of dNTP (10mM), 0.5 µl Advantage 2 polymerase, 1 µl of the CA gene specific primer, OoCAF1, and 1 µl of the 3' CDS II A primer and 19.0 µl sterile distilled water. The conditions of the PCR reaction consisted of an initial 1 min denaturation at 95°C followed by 25 cycles of 95°C 15 sec, 70°C 20 sec, 68°C 1 min. Reaction products were analyzed by TAE agarose gel electrophoresis visualized using 10 µg/ml ethidium bromide and cloned into pTOPO as previously described.

3.2.8 PCR Amplification of the Entire *Ostertagia ostertagi* Carbonic Anhydrase CDNA

Following the isolation of various cDNAs encoding for the *O. ostertagi* CA, a cDNA encoding for the entire CA enzyme was isolated from exsheathed L3 cDNA by PCR. The PCR reaction consisted of sSMART cDNA as template and the primer pair CASTRT / CDS II A was used. CDNA was prepared using the sSMART protocol as described above (2.7). The PCR conditions were an initial 1 min denaturation at 95°C followed by 25 cycles of 95°C 15 sec, 70°C 20 sec, 68°C 1 min. PCR products were analyzed by agarose gel electrophoresis as described previously.

3.2.9 Isolation of Recombinant Plasmid DNA Following Cloning

Colonies containing recombinant plasmids were identified by size using PCR as described above. These colonies were used to inoculate 5 ml cultures of LB media containing 100 µg / ml kanamycin. The inoculated cultures were grown overnight, shaking, at 37°C. This culture was then used to prepare a preparation of plasmid DNA. All preparations of plasmid DNA were prepared using the Qiagen spin miniprep kit (Qiagen, Valencia, CA). An overnight culture (1.5 ml) was centrifuged in an eppendorf tube at 12,000 rpm for 5 min. The bacterial pellet was resuspended in 250 µl of buffer P1 (100 µg/ml RNase a, 50 mM Tris/HCl, 100 mM EDTA, pH 8.0), then lysed through the application of 250 µl of buffer P2 (200 mM NaOH, 1%

SDS). Lysis was allowed to occur at room temperature for 3-5 minutes, then 250 µl of neutralization buffer (pH 4.8) was added and the solution was incubated on ice for 15 minutes. Following centrifugation, the supernatant was removed and applied to a spin column, contained within a microfuge tube. The column, within the tube, was centrifuged for 2 minutes at room temperature at 12,000 rpm and the DNA bound to the column. Following a wash of 500 µl of Qiagen wash buffer (80 mM KAc, 8.3 mM Tris-HCl, pH 7.5, 40 µM EDTA) the column was centrifuged one more time to remove any residual fluid and 50 µl of double distilled water was applied to the column. This was incubated at room temperature for 5 minutes and the DNA was eluted by centrifugation at 12,000 rpm for 5 min at room temperature. The concentration of the recovered plasmid DNA was determined by spectrophotometry.

3.2.10 Sequence Data Analysis

All sequencing was performed by BIOMMED (Louisiana State University School of Veterinary Medicine, Baton Rouge, LA). All sequence data were analyzed using the BLAST algorithm available at either the National Center for Biotechnology homepage (BLAST) (www.ncbi.nlm.nih.gov), the Wormbase home page (BLAST or BLAT) (www.wormbase.org) or the Nematode Net page (Nemablast) (www.nematode.net).

3.2.11 Isolation of *Ostertagia ostertagi* Actin

Searching of the NCBI genbank of *O. ostertagi* ESTs revealed a portion of a cDNA encoding for actin (Accession #AF052043). Based upon this sequence, two primers were designed to isolate a portion of actin from exsheathed *O. ostertagi* L3 cDNA. The primers used in the PCR were OoActF; 5'-ccg cac gct att ctc cgt ctt gat c-3' and OoActR; 5'-cgg aat cgc tag tt ccg aca gt-3'. The first three nucleotides in each primer were not part of the gene sequence but were added to increase the melting temperature (T_m) of the primers. These primers were used with exsheathed L3 cDNA produced using the sSMART™ cDNA synthesis protocol as

described previously. A PCR master mix was prepared that consisted of 2.5 µl 10X Advantage 2 PCR buffer, 1.0 µl of dNTP (10mM), 0.5 µl Advantage 2 polymerase, 1 µl of the OoActF and 1 µl of the OoActR primer, OoCAF1, and 1 µl of the 3' CDS II A primer and 19.0 µl sterile distilled water. The conditions of the PCR reaction consisted of an initial 1 min denaturation at 95°C followed by 25 cycles of 95°C 15 sec, 61°C 20 sec, 68°C 1 min. Reaction products were analyzed by TAE agarose gel electrophoresis and visualized using 10µg/ml ethidium bromide. PCR products were cloned and sequenced as described above.

3.2.12 Quantitative Analysis of Carbonic Anhydrase Expression During Exsheathment

The mRNA expression levels of CA were measured in *O. ostertagi* L3 at different times post exposure to the host rumen environment. L3 were cultured from the feces of infected steers as described previously (Chapter 1). The L3 were washed in distilled water and 4,500 L3 were aliquoted into individual dialysis bags (Product No. 146-502, Spectrum Laboratories, Inc., Rancho Dominguez, CA) dialysis bags were placed into the rumen of the cannulated steer, as described in Chapter 1 Section 2.5. The dialysis bags were removed at various times post exposure and the L3 were recovered from the bag. An aliquot of at least 100 larvae were kept and fixed in iodine to determine the percentage of exsheathed larvae. The rest of the larvae were rinsed by centrifugation in PBS and snap frozen as a dry pellet in liquid nitrogen. Total RNA was later isolated from these L3 using the methods described above. Reverse transcription was also carried out as per the above protocol and the cDNA was used as a template for the quantitation of CA expression during exsheathment.

Quantitation of CA mRNA expression during exsheathment was carried out using the Applied Biosystems Sequence Detection System, 7700 (Taqman). The expression levels of actin mRNA were also analyzed in these samples, as a means of standardizing CA mRNA expression to the expression of a housekeeping gene.

All primer and probes utilized in the Taqman reactions were designed using the Primer Express software (Applied Biosystems) and are listed in Table 3.2. All primers were left unlabeled. Probes used in the Taqman reactions were labeled with the reporter dye FAM (6-carboxyfluorescein) and the quencher dye TAMRA (6-carboxytetramethyl-rhodamine).

All Taqman reactions were carried out using Universal PCR Master Mix as per manufacturers directions. Each reaction consisted of 25 μ l Applied Biosystems Master Mix, 17 μ l double distilled water, 1 μ l sense primer (20 μ M), 1 μ l antisense primer (20 μ M), 1 μ l Taqman probe (10 pmoles/ μ l) and 5 μ l cDNA template. Reaction conditions were as recommended by Applied Biosystems, namely; 50°C 2 min, 95°C, 10 min, then 40 cycles of 95°C 15 sec and 60°C 1 min. All PCR analyses were carried out in duplicate.

Cycle threshold (CT) values were obtained for CA and actin for each L₃ cDNA sample. Standard curves were constructed using plasmids containing the genes encoding either actin or CA. For each plasmid, sufficient DNA was diluted in double distilled water to provide 10⁻¹⁵ moles of plasmid / μ l. This solution was then serially diluted in logs, so that the most dilute solution contained 10⁻²³ moles / μ l. The most dilute solution was designated 1, according to the molecular weight of 1 mole of any solution being equivalent to Avagadros number, or 6.02 x 10²³ moles. The most concentrated solution, being 8 logs more concentrated, was designated 1x10⁸. A standard curve was constructed for both actin and CA in this manner. Cycle threshold (CT) values were obtained for each point on the standard curve as well as each cDNA sample.

Linear regressions were carried out on both the actin and CA CT values as described in the ABI PRISM 7700 Sequence Detection System User Bulletin #2 (Applied Biosystems). The interpolated values for CA were normalized against those obtained for actin mRNA by dividing the value for CA by that obtained for actin. In this way, the data for CA mRNA expression levels is expressed per actin molecule. The data presented are the means of between two and

three cDNA samples obtained from larvae exsheathed in the rumen on different days \pm standard error.

Table 3.2. Sequences of the primers and probes used to quantitate carbonic anhydrase and actin mRNA levels in *Ostertagia ostertagi* L3 during the process of exsheathment. All sequences are written in the 5'-3' orientation. Primers were unlabeled. Probes were labeled with the reporter dye FAM (6-carboxyfluorescein) and the quencher dye TAMRA (6-carboxytetramethylrhodamine).

GENE	SENSE PRIMER	ANTISENSE PRIMER	PROBE
CA	cga ctt tcg cgc aac ga	ccg aat cat tgc ctt cac gt	ggc aac gcc gtc tgg ctg
Actin	gac cca gat cat gtt cga gac ct	cac cat ctc cag agt cga gca	cgt cgc tat cca ggc tgt gct ctc c

3.3 Results

3.3.1. Initial Isolation of *Ostertagia ostertagi* Carbonic Anhydrase Gene

The RNA recovery procedure used on exsheathed and sheathed *O. ostertagi* L₃ yielded, on average, 0.3 ng total RNA / L₃ processed. Reverse transcription was carried out on total RNA recovered from *O. ostertagi* L₃ to produce cDNA for use as a PCR template to isolate a CA gene. Sequences of six different CA genes from *C. elegans* are described on the website www.wormbase.org. The translated sequence of the cah-6 gene from *C. elegans* was used to search an *O. ostertagi* EST database available at www.Nematode.net using the nemablast search algorithm. This search identified a 547 bp *O. ostertagi* EST (NCBI Accession Number BQ097482) that had a 70% amino acid sequence homology with the *C. elegans* CAH-6 sequence (Figure 3.1).

3.3.2 Isolation of an Initial *Ostertagia ostertagi* Carbonic Anhydrase Transcript

PCR primers (Table 3.1) were designed based on the nucleic acid sequence of the identified *O. ostertagi* CA EST to isolate the full-length transcript of the gene. The location of the primers

NSL1, oligo dT, CDS II A, two sense primers and two antisense primers in relation to the nucleic and amino acid sequence of EST is illustrated in Figure 3.2.

Expect = 7.1e-71, P = 7.1e-71

Identities = 128/182 (70%)

Positives = 150/182 (82%)

Oo EST	KLDSL	GKVLH	V	SGL	GRYP	SP	IDIV	PVIT	CF	GEHL	QNAE	FR	VE	YHNT	GD	FRAM	NS	GNA	VWL																																									
CAH-6	KLDSL	GKVLN	V	PSL	GR	TQ	SP	IDIV	PVIT	AF	GEHL	QNAH	FE	VT	YES	TGE	FKAV	NDGNS	IWL																																									
Oo EST	L	R	E	G	N	D	S	E	L	A	V	S	F	L	P	E	E	Q	Y	H	L	D	S	I	T	W	H	G	T	E	P	M	N	G	S	E	H	T	I	G	G	V	G	Y	A	G	E	L	H	L	I	H	R	N	T	R	F	P	T	
CAH-6	M	R	E	G	N	S	S	E	L	A	I	S	F	L	P	E	E	Q	Y	H	L	D	A	V	N	F	H	W	A	T	E	P	M	N	G	S	E	H	T	I	G	G	V	G	Y	A	G	E	M	H	L	I	H	R	N	T	R	F	A	T
Oo EST	T	E	L	A	L	K	Q	P	N	G	V	L	A	I	A	I	F	L	N	E	S	H	D																																					
CAH-6	M	A	D	A	L	K	Q	P	N	G	V	I	A	I	A	V	F	L	N	E	S	H	D	D	N	A	V	F	S	P	L	I	N	L	L	P	Q	V	I	Y	K	G	S	E	C	K	L	C	S	F	D	F	Q	T	F	F	P	V	A	E

Figure 3.1. Alignment of the *Ostertagia ostertagi* expressed sequenced tag (Oo EST) (Genbank # BQ097482) and the *Caenorhabditis elegans* cah-6 protein, CAH-6 (Genbank # NM_058788.1.). Homologous amino acid residues are indicated by blue. Similar amino acid residues are indicated by yellow. Expect, the probability that the match occurred by chance. Identities, the number of identical amino acids shared by the two sequences. Positives, the number of conserved amino acids (similar in nature) between the two sequences.

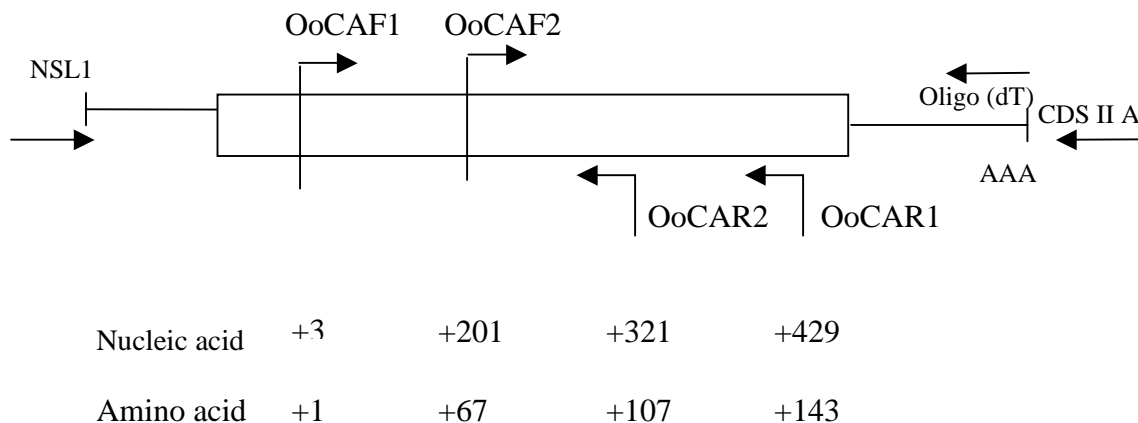


Figure 3.2. Primer location relative to the nucleic and amino acid sequence of the *Ostertagia ostertagi* CA expressed sequence tag (BQ097482).

A PCR product of 427 bp was obtained following the PCR using primers OoCAF1 and OoCAR1. This product was subsequently blunt cloned and sequenced according to the protocols described above. A blast search was carried out on the translated sequence using the NCBI search algorithm as previously described. This search revealed that the PCR product obtained from the OoCAF1 and OoCAR1 primer pair was homologous to both the *O. ostertagi*

EST sequence available on the internet as well as CA's from several organisms, including *C. elegans*.

3.3.3 Isolation of the 5' End of Carbonic Anhydrase

The majority of nematode mRNA's have a spliced leader (SL) sequence post-transcriptionally added to their 5' end. Although the portion of mRNA's containing a SL in *O. ostertagi* is not known, it is estimated that 70-90% of *A. suum* mRNAs and 70% of *C. elegans* mRNAs contain a SL sequence (Blumenthal 1997), (Nilsen 1993). Given the high percentage of SL mRNAs in these two nematode species, the SL sequence primer, designated NSL1 (Table 3.1), was used to obtain the 5' of the CA gene from *O. ostertagi*. A PCR reaction using the NSL1 and OoCAR1 primer pair resulted in a 633 bp product. This product was subsequently cloned and sequenced according to previously described procedures. Sequence analysis of this product revealed a start codon (ATG). The nucleotide sequence showed high homology (98% identity, 98% similarity) to the original *O. ostertagi* EST [BQ097482]. An alignment of the nucleic acid sequence of the PCR products obtained with NSL1 / OoCAR1 and OoCAF1 / OoCAR1 confirmed that each primer pair was amplifying the same cDNA.

3.3.4 Isolation of the 3' End of Carbonic Anhydrase

A PCR reaction was carried out with exsheathed *O. ostertagi* L₃ cDNA produced with the sSMART cDNA synthesis procedure as template and primer pair OoCAF1 / CDSIIA for amplification of the 3' end of the CA. This reaction resulted in a 1600 bp product. Amplicons from each primer pair were cloned and sequenced according to procedures previously described. Analysis of the nucleotide sequence confirmed that the 3' end of the CA gene had been recovered, including a large portion of 3' untranslated region (UTR). A stop codon (TAG) and a polyadenylation signal tail were both found in the sequence. Blast analysis with the cDNA previously isolated using OoCAF1 and OoCAR1 showed 98% identity between the two

sequences, confirming the two cDNAs were encoding for the same CA. Figure 3.3 shows a schematic of the cDNA clones isolated and their relationship to each other in forming the entire cDNA encoding for *O. ostertagi* cDNA.

3.3.5 Determination of the Entire CDNA Sequence of the *O. ostertagi* Carbonic Anhydrase

The entire CA gene sequence was determined by PCR using the CAsStart / CDS II A primer pair and sSMART cDNA as a template. Figure 3.4 shows the entire DNA and putative amino acid sequence of the CA isolated from *O. ostertagi* L₃. This sequence has been submitted to Genbank under the accession number AY621380. Appendix 1 shows the entire cDNA sequence, including the 3'UTR. The two sequences that showed the greatest homology were CAs from *Caenorhabditis briggsiae* and *C. elegans*. Following these, the next three genes most closely resembling the *O. ostertagi* CA were all variants of human CA III. The sixth gene to show homology to the cloned CA was human CA VII. Table 3.3 summarizes the first 5 blast results and shows the percent identity shared between the cloned *O. ostertagi* gene and other CAs.

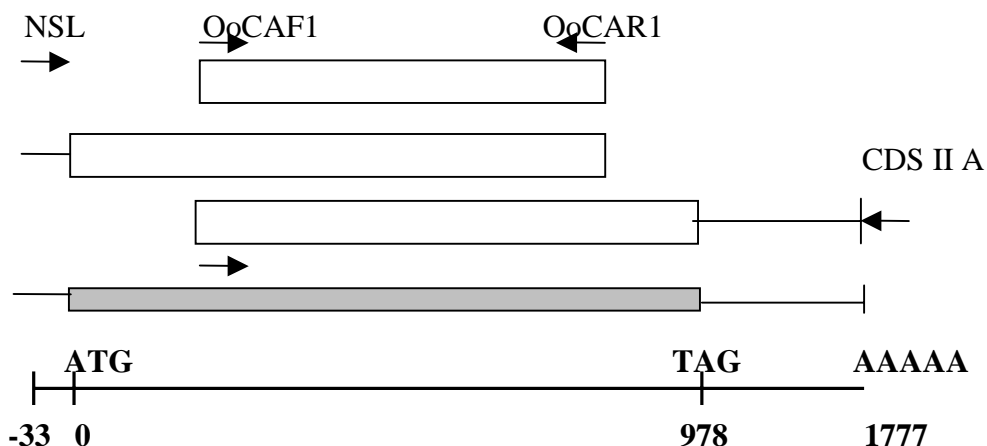


Figure 3.3. Schematic diagram of the cDNA clones amplified by PCR using multiple primer pairs. The first cDNA was isolated using OoCAF1 / OoCAR1. The second was amplified using NSL1 / OoCAR1. The carboxy terminus of the CA was amplified using OoCAF1 / CDS II A. The joined sequences represent the entire *Ostertagia ostertagi* carbonic anhydrase cDNA (grey box).

```

      10      20      30      40      50      60      70      80      90     100
1  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
   ATGGCGTTTCTCAGCCAATCTCAGGTCATTTTCAGACATTAAAGGAAAAACAGCCACGATGGCTGAAGGAGCGCCGCAATCGGAGCACATCTGCAGT
   M A F L S Q L S G H F Q T L K E K T A T M A E G A P A I G A H L Q

      110     120     130     140     150     160     170     180     190     200
101 CACTAAAAGTCGGTGCCCAAGTTGATCGAAAGTGCCTTGAATGCTAAAAACGGACTTCTCAATAGGGTGAAACTCGACTCACTTGGCAAAAGTACTGCA
   S L K V G A P K L I E S A L N A K N G L L N R V K L D S L G K V L H

      210     220     230     240     250     260     270     280     290     300
201 CGTGTCCGGTTTGGGTCGCTACCCATCACCTATTGATATCGTGCCGGTCATCACCTGCTTCGGAGAACATCTGCAAAATGCCGAGTTTCCGGTTCGAGTAC
   V S G L G R Y P S P I D I V P V I T C F G E H L Q N A E F R V E Y

      310     320     330     340     350     360     370     380     390     400
301 CACAATCTGGCGACTTTCGCGCAACGAATTCTGGCAACGCGCTTGGCTGCTAGCTGAAGGCAATGATTTCGAGTTTGGCGGTTCGTTTCTGCCAGAGG
   H N T G D F R A T N S G N A V W L L R E G N D S E L A V S F L P E

      410     420     430     440     450     460     470     480     490     500
401 AGCAGTATCATCTGGACTCAATCACATGGCACTGGGGAACGGAGCCATGAATGGCTCCGAACACACCATAGGAGGGTTGGATACGCCGCGCACTGCA
   E Q Y H L D S I T W H W G T E P M N G S E H T I G G V G Y A G E L H

      510     520     530     540     550     560     570     580     590     600
501 TCTGATCCATCGCAATACCGCTTCCCGACGATGGAGTTGGCGCTGAAACAGCCGAACGGAGTGTCTCGCATCGCCATCTTCTCAATGAGTTCACAGAC
   L I H R N T R F P T M E L A L K Q P N G V L A I A I F L N E S H D

      610     620     630     640     650     660     670     680     690     700
601 GAAAATCCAGCAATTACTCCGTTTATTGAGTTGTTACCGAATATCACCTACAAGGGCAACGAGGTCGCGCTCGGCAGTTTCAACTTCGGCGCACTTTTTC
   E N P A I T P F I E L L P N I T Y K G N E V R V G Q F N F G A L F

      710     720     730     740     750     760     770     780     790     800
701 CATCTCCAGAGAAGACTAAGGAGTTTGGATGTACGAAGGATCGGAGACGGTAGAGCCTTCCGCGAGACAGTTTCACTGGGCTTGTGTTCCGCTCAGCTGT
   P S P E K T K E F W M Y E G S E T V E P F R E T V Q W L V F R S A V

      810     820     830     840     850     860     870     880     890     900
801 GCCAATCAGCTCATATCAGCTTGATCGTCTACGTGAGGTTTCGCTCTGGTGGCTACGACGAGGAGCGCGAGACACCGATGGAGCCGATTCTGTCACACAG
   P I S S Y Q L D R L R E V R S G G Y D E E R E T P M E P I R P P Q

      910     920     930     940     950     960     970
901 CCACCAAACTCGCGCAGCGTCGTGTGTAGTTTTCGCGAGCGCAGCCGCGCTCCCGATCTTGGATTCAACAACAGTAG
   P P N S R S V V C S F R S A A G A P D L G F N K Q *

```

Figure 3.4. Entire DNA and putative amino acid sequence of the carbonic anhydrase isolated from *Ostertagia ostertagi* third stage larvae.

Figure 3.6 shows an alignment of the *O. ostertagi* CA amino acid sequence with the *C. elegans* homolog and the human CA III. Active site residues and zinc binding histidine residues are indicated.

3.3.6. Isolation of *Ostertagia ostertagi* Actin

A PCR reaction was carried out on *O. ostertagi* exsheathed L₃ in attempt to isolate a portion of the cDNA encoding for actin. The primers were based on the *O. ostertagi* actin sequence (Accession # AF052043) available on the NCBI genbank. The gene sequence was

needed for use as a reference gene for quantitative analysis of CA gene expression. The PCR reaction resulted in the recovery of a 252 bp product. This product was cloned and sequenced.

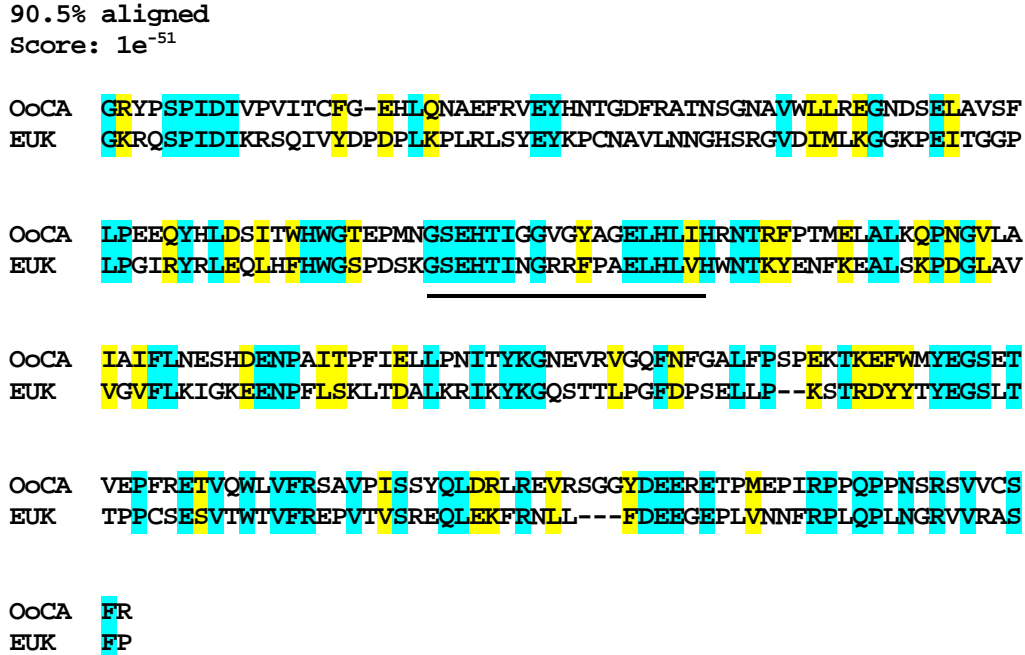


Figure 3.5. Alignment of the *Ostertagia ostertagi* carbonic anhydrase (OoCA) with the eukaryotic consensus sequence (EUK). The underlined amino acid residues comprise the signature carbonic anhydrase motif. The carbonic anhydrase motif of the *O. ostertagi* cDNA is 90.5 % aligned with the eukaryotic consensus sequence. The score represents the probability that the sequence homology would occur by random occurrence. Aqua shading indicates identical amino acid residues. Yellow shading indicates similar amino acid residues.

Table 3.3. First five results obtained from blasting *Ostertagia ostertagi* carbonic anhydrase against the translated nucleic acid database. Score, the probability that the homology between the two genes occurs by random occurrence. % Id / Sim, the percentage of amino acids that are either identical (Id) or similar (Sim) between the isolated CA and the cDNAs in the NCBI database. *C. briggsiae*, *Caenorhabditis briggsiae*. *C. elegans*, *Caenorhabditis elegans*.

Organism	Gene	Accession No.	Score	% Id / Sim
<i>C. briggsiae</i>	unknown	CAE66652	e^{-120}	67 / 78
<i>C. elegans</i>	Cah-6	NM_058788	e^{-120}	66 / 78
Human	CA III	1205233A	$2e^{-28}$	38 / 55
Human	CA III	NP_005172.1	$2e^{-28}$	38 / 55
Human	CAIII	AAH04897	$2e^{-28}$	38 / 55

3.3.7 Amino Acid Composition of the Essential Hydrophobic Core and Active Sites of *Ostertagia ostertagi* Carbonic Anhydrase

The amino acid residues found within the hydrophobic core and the active site residues are important features of the CA genes. To investigate the amino acid composition of these features within the *O. ostertagi* CA two tables were constructed to compare the amino acid residues in these areas with those of *C. elegans* and human CAIII.

OoACT	MVGMGQKDSYVGDEAQSKRGILTLKYP ^{IEHGIVTNWDDMEKIWHHTFYNG} LRVAPEEHPV
PgACT	MVGMGQKDSYVGDEAQSKRGILTLKYP ^{IEHGIVTNWDDMEKIWHHTFYNELRV} AP ^{EEHPV}
CeACT	MVGMGQKDSYVGDEAQSKRGILTLKYP ^{IEHGIVTNWDDMEKIWHHTFYNELRV} AP ^{EEHPV}
OoACT	LLTEAPLNPKANREKMTQIMFETFNTPAMYVAIQAVLSLYASGR ^{TTG} VVLDSGDGV ^{THTV}
PgACT	LLTEAPLNPKANREKMTQIMFETFNTPAMYVAIQAVLSLYASGR ^{TTG} VVLDSGDGV ^{THTV}
CeACT	LLTEAPLNPKANREKMTQIMFETFNTPAMYVAIQAVLSLYASGR ^{TTG} VVLDSGDGV ^{THTV}
OoACT	PIYEGYALPHAILRLDLAGRGLTDYLMKILTERGYSF ^{TTTAEREIVRDIKEKLCYVALDF}
PgACT	PIYEGYALPHAILRLDLAGRDLTDYLMKILTERGYSF ^{TTTAEREIVRDIKEKLCYVALDF}
CeACT	PIYEGYALPHAILRLDLAGRDLTDYLMKILTERGYSF ^{TTTAEREIVRDIKEKLCYVALDF}
OoACT	EQEMATAASS
PgACT	EQEMATAASS
CeACT	EQEMATAASS

Figure 3.7. BLAST alignment of *Ostertagia ostertagi* actin amino acid sequence (OoACT) with that of *Panagrellus redivivus* (PgACT) (AAQ89578.1) and *Caenorhabditis elegans* (CeACT) (NP_505817.1). Blue shading indicates identical amino acid residues.

Table 3.4 shows the amino acid residues that comprise the hydrophobic core of the CAs. Of the 34 residues, *O. ostertagi* shares 14 with human CAIII. In addition, a further 10 amino acids are conserved, or possess similar biochemical properties. Only 10 of the entire 34 residues analyzed are completely different within *O. ostertagi*, compared to human CAIII. Of the 10 residues on OoCa that show no homology to human CAIII, 9 are either identical or similar to the respective amino acids found in CAH-6.

Table 3.5 shows the amino acid composition of the active site of the *O. ostertagi* CA, compared with the active sites of human CAIII and CAH-6. Of the 16 amino acid residues found within the active site of these enzymes, 4 are identical between OoCA and human CAIII. A further 6 amino acid residues are conserved, or possess similar biochemical characteristics. Of

the 6 amino acids in the OoCA active site that do not share any homology with human CAIII, there are 5 that are identical in the active site of CAH-6, suggesting these coding differences may be nematode specific.

Table 3.4. The amino acid composition of the *Ostertagia ostertagi* (OoCA) hydrophobic core, compared with that of *Caenorhabditis elegans* (CAH-6) and human carbonic anhydrase III (HCAIII). Residue numbering is based on HCAIII. Bold residues are identical and italicized residues are similar, between OoCA and HCAIII. Similar amino acids share the same biochemical characteristics, including charge and hydrophobicity.

RESIDUE	OoCA	CAH-6	HCAIII	RESIDUE	OoCA	CAH-6	HCAIII
41	<i>Phe</i>	Phe	<i>Trp</i>	148	Asn	Asn	Leu
49	Val	Val	Val	157	Thr	Ser	Phe
51	Tyr	Tyr	Tyr	160	Ile	Ile	Phe
59	<i>Ala</i>	Ala	Ile	161	Glu	Asn	Leu
66	<i>Val</i>	Ile	<i>Cys</i>	164	Leu	Leu	Leu
68	<i>Leu</i>	Leu	<i>Val</i>	167	Ile	Val	Ile
70	Arg	Arg	Phe	176	Val	Leu	Phe
79	<i>Val</i>	Ile	<i>Leu</i>	179	Phe	Phe	Phe
90	Leu	Leu	Leu	184	Leu	Phe	Leu
93	Ile	Val	Phe	185	Phe	Phe	Phe
95	Trp	Phe	Leu	210	Leu	Val	Leu
97	Trp	Trp	Trp	212	Phe	Ile	Leu
118	Leu	Met	Leu	216	<i>Val</i>	Leu	<i>Met</i>
120	Leu	Leu	Leu	218	<i>Ile</i>	Ile	<i>Val</i>
122	His	His	His	223	<i>Leu</i>	Leu	<i>Met</i>
144	<i>Ile</i>	Ile	<i>Val</i>	226	Leu	Leu	Leu
146	<i>Ile</i>	Val	<i>Gly</i>	229	<i>Val</i>	Val	<i>Leu</i>

Table 3.5. Amino acid composition of the active site of *Ostertagia ostertagi* carbonic anhydrase (OoCA), compared to those of *Caenorhabditis elegans* carbonic anhydrase (CAH-6) and human carbonic anhydrase III (HCAIII). Bold text indicates amino acid residues that are identical between OoCA and HCAIII. Italics indicates amino acids that are similar / conserved between OoCA and HCAIII.

RESIDUE	OoCA	CAH-6	HCAIII	RESIDUE	OoCA	CAH-6	HCAIII
HYDROPHILIC				HYDROPHOBIC			
7	<i>Phe</i>	Phe	<i>Tyr</i>	91	Asp	Asp	Arg
62	<i>Ser</i>	Asp	<i>Asn</i>	121	<i>Ile</i>	Ile	<i>Val</i>
64	Asn	Asn	Lys	131	Met	Met	Phe
67	Trp	Trp	Arg	135	Leu	Leu	Leu
92	<i>Ser</i>	Ala	<i>Gln</i>	143	<i>Ile</i>	Ile	<i>Val</i>
106	Glu	Glu	Glu	198	Glu	Glu	Phe
199	Thr	Thr	Thr	207	<i>Val</i>	Val	<i>Ile</i>
200	Val	Thr	Thr	209	Trp	Trp	Trp

3.3.8 Quantitative Analysis of Carbonic Anhydrase Expression During Exsheathment

In several separate exsheathment studies, aliquots of 3,000 *O. ostertagi* L₃ were exposed to the rumen for various time-points to initiate exsheathment. These studies were carried out while the cannulated steer was on a grass diet and also while on a grain diet. The time-points of exposure and number of exsheathment studies conducted while the steer was on the each diet is listed in Table 3.6.

3.3.8.1 Grass Diet

Figure 3.8A illustrates the CA expression pattern in *O. ostertagi* L₃ exposed to grass-derived rumen contents. Copy units (level of expression) of CA increased to levels 3 times greater than non-exposed L₃ 5 min following exposure to the rumen. Maximum expression, 4.5

fold greater than non-exposed, was observed 15 min post-exposure. CA expression levels remained between 2 and 4 fold greater than non-exposed *L*₃ between post-exposure times of 30 min to 120 min. The level of expression dropped precipitously after 420 min relative to the 120 min time-point, but was still elevated relative to non-exposed larvae.

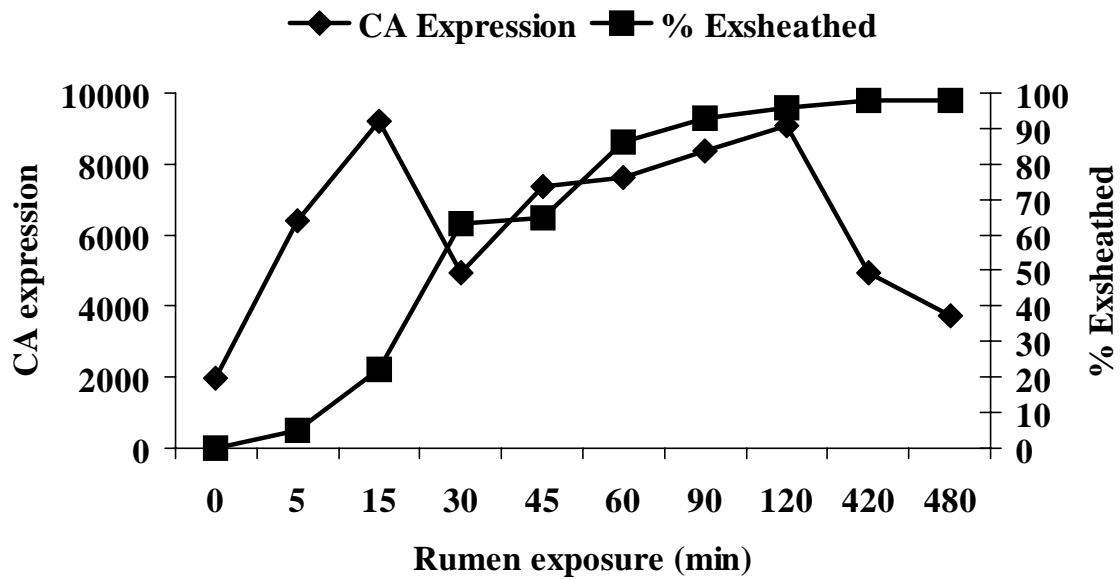
Table 3.6. The number of exsheathment studies carried out to quantitate carbonic anhydrase expression in *Ostertagia ostertagi* third stage larvae exposed to the rumen of a cannulated steer while the steer was on a grass or grain ration.

Diet	Exposure to Rumen (min)	Experimental Replicates
Grass	0	2
	5	1
	15	1
	30	2
	45	1
	60	2
	90	1
	120	2
	420	2
	480	1
Grain	0	2
	60	2
	120	4
	240	4
	360	2

3.3.8.2 Grain Diet

The CA expression pattern in *O. ostertagi* *L*₃ exposed to grain-derived rumen content is illustrated in Figure 3.8B. CA expression levels remained relatively unchanged until 360 min post-exposure, at which time a 3.5 fold increase in CA expression was observed relative to non-exposed larvae.

A



B

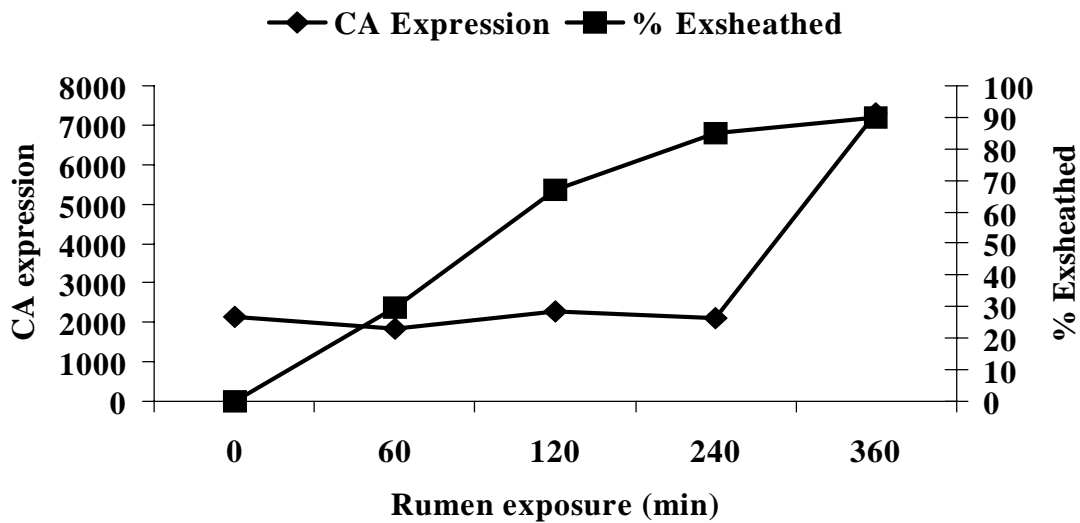


Figure 3.8. Expression of *Ostertagia ostertagi* carbonic anhydrase in relation to the exsheathment kinetics of *O. ostertagi* L₃ on (A) grass diet or (B) grain diet.

3.4 DISCUSSION

The aim of this study was to isolate a CA gene from *O. ostertagi* and subsequently describe the pattern of CA expression during the exsheathment process. The first of these

objectives was accomplished in the isolation of a CA isoform from *O. ostertagi* (OoCA) with a deduced amino acid composition showing greatest homology to the *C. elegans* CAH-6. Of all the isoforms reported from humans, the OoCA conforms more closely to the human CAIII (HCAIII) isoform. In addition, the gene shows 90.5% alignment with the eukaryotic consensus sequence motif for CAs. Given these similarities, the gene was termed OoCAIII.

Initially, it was difficult to assign an isoform designation to the OoCA due to the high homology of the deduced amino acid sequence with CAH-6. CAH-6 is described in Wormbase as being more similar to human CAVII. Blast analysis of the OoCA revealed a greater homology with HCAIII rather than HCAVII. While OoCAIII and CAH-6 share strong homology over their entire coding sequence, the highest degree of homology is observed within the 3' (carboxy) end of the gene. Indeed, if the 3' end of OoCAIII is blasted against the NCBI database, it shows stronger homology to HCAVII than to HCAIII. The differences in the amino acid composition of the 5' end of OoCAIII and CAH-6 are sufficient for these two molecules to be classified as different isoforms. The crystal structure of HCAIII is currently undetermined. However, the crystal structures of bovine CAIII (BCAIII) and rat CAIII (RCAIII) are known. A comparison of the amino acid composition of the active sites and hydrophobic core of RCAIII and BCAIII with the amino acid sequence of HCAIII, reveals that HCAIII is 100% (16/16 identical residues) and 94% (32/34 identical residues) homologous in these two regions. Given the relatively high homology of the OoCA with HCAIII resulting from the GenBank Blast analysis, it is reasonable to compare the amino acid residues from OoCA that align within the putative active site and hydrophobic core of HCAIII. Comparison of the amino acids from OoCA that align within the hydrophobic core of HCAIII, RCAIII, and BCAIII, revealed that 70.5% are identical or share similar biochemical properties. In addition, 62.5% of the amino acids from OoCA are identical or similar to those within the active sites of RCAIII, BCAIII, and HCAIII.

The hydrophobic core residues are important for orienting the histidine ligands in a conformation that favors zinc binding and destabilizes geometries that favor other metals. The active site residues form a 15-angstrom wide, 15-angstrom deep cavity at the bottom of which the zinc ion is ligated by histidines 94, 94, and 119 (Tripp 2001). This cavity is important not only in binding the active zinc molecule but also for orienting water molecules that reestablish the zinc-bound hydroxyl group necessary for the hydration of CO₂ (Eriksson 1991).

All mammalian α -CA's reported to date, contain three histidine (H) residues at positions 94, 96, and 119 that are responsible for binding zinc. For OoCAIII, only two of the histidines (H) are present at the comparable positions of 96 and 119. The histidine (H) at position 94 is, interestingly a threonine (T). A histidine (H) is present, however, at position 89. A similar case is seen in CAH-6. Whether this histidine (H) would confer a tertiary structure leading to participation in zinc binding is not known. However, the presence of a similar sequence change in CAH-6 supports the theory that this may, indeed, be a nematode-specific CA characteristic. Further analysis of other CAs from *O. ostertagi*, as well as from other parasitic and non-parasitic nematodes, would help answer this question.

Interesting differences within both the putative hydrophobic core and active site regions of the OoCAIII were observed relative to those of RCAIII, BCAIII, and HCAIII. For example, in the hydrophobic core of HCAIII, RCAIII, and BCAIII, amino acid 157 is a phenylalanine (F), in OoCAIII it is a threonine (T) (position 208 of OoCAIII sequence) and a Ser (S) in cah-6. This substitution may represent a potential phosphorylation site unique to nematode CAIII within the hydrophobic core (Eriksson 1991). In addition, two cysteine (C) residues at positions 183 and 188 in RCAIII, BCAIII, and HCAIII are known in RCAIII to form disulfide linkages with glutathione in response to oxidative stress through a process of glutathiolation. Glutathiolation of the cysteines protects these residues from irreversible oxidation (Cabiscol 1996). Protection of

the cysteines is important as these two residues confer phosphatase activity to the CAIII, a property unique to this isoform. The residues present in OoCAIII at these comparable locations are alanine (A) and threonine (T), respectively. Perhaps the glutathione-binding site at position 188 in mammals is an activation site or reactive site in nematode CAIII, as a threonine (T) is also present in the comparable position in CAH-6 of *C. elegans*. OoCAIII does however, possess two cysteine (C) residues, but these are located at positions 86 and 308 of the OoCAIII sequence. The changes in the positioning of these cysteine residues may mean that (1) OoCAIII and CAH-6 exhibit a difference in protein folding compared to HCAIII, RCAIII and BCAIII, and (2) that OoCAIII and CAH-6 are still able to form an internal disulfide bridge.

A comparison of amino acids of OoCAIII that align with the active site residues of HCAIII, RCAIII, and BCAIII reveal two interesting substitutions in the OoCAIII. At positions 62 and 92 of HCAIII, RCAIII and BCAIII, the active site residues are aspartic acid (D) and glutamine (Q), respectively. However, in OoCAIII these residues are both serine (S). These two residues may also represent novel activation sites within the OoCAIII as serine and threonine residues are well known for their role as active sites on protein kinases and phosphatases, playing significant roles in cell signal transduction pathways.

Previous reports on the biochemistry of exsheathment of related trichostrongylid nematodes suggest a role for CA in exsheathment (Rogers 1960; Rogers 1962; Rogers 1976; Davey 1982) although a gene encoding a CA has not previously been isolated from a trichostrongylid nematode. The hypothesized role of CA in exsheathment was originally to transmit a host signal to the infective L₃ that subsequently initiates exsheathment. Biochemical data suggests a rapid exchange of one or more of the components within the equation $\text{H}_2\text{O} + \text{CO}_2 \leftrightarrow \text{H}_2\text{CO}_3 \leftrightarrow \text{H}^+ + \text{HCO}_3^-$ serves as the stimulus for exsheathment. A CA isoform with a high catalytic rate would more likely be involved in this role. CAIII, however, has one of the

slowest catalytic rates of the active CA isoforms (Khalifah 1991) and would theoretically not be the optimum isoform to fulfill the role as an initiator of exsheathment.

The expression pattern of CA in *O. ostertagi* observed during the time course of exsheathment on the two different diets provides further evidence that this isoform may not function in initiation of exsheathment. Under both sets of conditions (grass and grain) the expression level of OoCAIII did not increase until L₃ had already begun exsheathing. For example, when L₃ were exposed to grain-derived rumen contents for 1 hr, approximately 32% of them exsheathed, and 88% of L₃ were exsheathed by 4 hr. However, little to no change in CA expression was observed in larvae at either 1 hr or 4 hr, relative to non-exposed larvae. CA expression was increased 4 fold following 6 hr exposure and this correlated with maximum exsheathment (90%), suggesting this isoform may, indeed, be activated during the exsheathment process.

Under grass-derived rumen conditions the expression pattern of OoCAIII more closely mimics the exsheathment kinetics of *O. ostertagi* L₃. When larvae are exposed to grass-derived content, they exsheath more quickly, compared to those exposed to grain-derived content, reaching 88% exsheathment within the first hour. At this time, OoCAIII expression is increased approximately 3.5 fold, compared to levels found in non-exsheathed larvae. Larvae exposed to grass-derived content continue to exsheath over time, with maximum exsheathment reached by 2 hr. Following this time period very few L₃ continue to exsheath. The expression of OoCAIII also continues to increase over time, reaching maximum levels at 2 hr. After this time, however, levels of OoCAIII decrease and are almost equal to pre-exsheathment levels by 8 hr. Under these conditions, then, it is possible that OoCAIII is involved in the exsheathment of *O. ostertagi* larvae in some capacity.

The data collected in these experiments suggest it is doubtful that this isoform functions as an initiator of exsheathment. However, based on the role of CAIII in mammalian systems, this isoform may serve an important role in maintaining larval survival during the exsheathment process by maintaining intracellular pH homeostasis (Spitzer 2002), and may also participate in signaling events initiated by potential high oxidative stress (Cabiscol 1996) experienced by *O. ostertagi* larvae during exsheathment.

The difference in OoCAIII expression in *O. ostertagi* L₃ exposed to grass-derived or grain-derived rumen contents is interesting. Based on what is known about rumen physiology, grass-derived (high roughage) rumen contents have significantly higher levels of HCO₃⁻ and H₂CO₃ (Dehority 2003). Recalling that carbonic anhydrase mediates a reversible reaction, CO₂ + H₂O \rightleftharpoons H₂CO₃ \rightleftharpoons HCO₃⁻ + H⁺, an influx of HCO₃⁻ would theoretically provide increased substrate for the reverse reaction and result in an increase in intracellular water and CO₂. There are several potential physiological consequences to this reaction. An increase in intracellular CO₂ theoretically would initiate a hypoxic response. In addition the increase in intracellular HCO₃⁻ could also initiate developmental pathways via soluble adenylyl cyclase signaling discussed in Chapter 1 of this work. A transient increase in HCO₃⁻ and consequently, increased CO₂, due to the activity of a CA, could also potentially increase the intracellular H⁺ ion diffusion coefficient leading to a spatially rapid wave of intracellular acidosis. The intracellular mobility of H⁺ ions in rabbit ventricular myocytes was increased 300 fold following local diffusion of CO₂/ HCO₃⁻. The increased H⁺ mobility was reported to be due to the activity of a CA. (Spitzer 2002).

The rumen environment resulting from a grain diet would theoretically have significantly lower levels of HCO₃⁻ due primarily to the significant decrease in salivation by the host. The consequences of this may be a delay or inefficiency of HCO₃⁻ or H⁺ ion signaling cascades. If these cascades serve as molecular switches for downstream transcriptional events leading to

development, then it is likely that initiation of development would be delayed. The delayed increase in OoCAIII expression in larvae exposed to grain-derived rumen contents, then, supports a role for this molecule in larval development following exsheathment.

The expression profile of OoCAIII in *O. ostertagi* larvae exposed to grass-derived rumen content also supports a role for OoCAIII immediately following exsheathment. Under these conditions larvae begin to exsheath within 5 min following exposure to the rumen. Similarly, OoCAIII expression peaks at 15 min post-exposure, further suggesting OoCAIII is up-regulated following exsheathment.

In conclusion, the functional role of this CA in the exsheathment process of *O. ostertagi* is not currently known. However, the fact that CA was differentially expressed and, more specifically, highly expressed under conditions conducive to optimal exsheathment, indicates that this CA is associated at some level with the exsheathment process or developmental events downstream of the exsheathment. It is also encouraging that the OoCAIII is similar enough to mammalian CA's to suggest it is functionally active, but may also possess structural differences that would allow the development of a nematode specific CAIII antagonist that could serve to prevent development following exsheathment.

3.5 REFERENCES

- Blumenthal, T. and K. Steward (1997). RNA Processing and Gene Structure. *C. elegans*_II. D. L. Riddle, T. Blumenthal, B.J. Meyer, J.R. Press. Plainview, Cold Spring Harbor Laboratory Press: 117-146.
- Bonner, T.P. and M. Buratt. (1976). The effect of actinomycin-D on development and infectivity of the nematode *Nippostrongylus braziliensis*. *International Journal for Parasitology* **6**: 289-294.
- Cabiscol, E. and R. Levine (1996). The phosphatase activity of carbonic anhydrase III is reversibly regulated by glutathiolation. *Proceedings of the National Academy of Science* **93**: 4170-4174.
- Davey, K.G., R.I. Sommerville and W.P. Rogers (1982). The effect of ethoxzolamide, an analogue of insect juvenile hormone, nor adrenaline and iodine on changes in the optical

- path difference in the excretory cells of oesophagus during exsheathment in *Haemonchus contortus*. *International Journal for Parasitology* **12**(6): 509-513.
- Davey, K.G. and W.P. Rogers (1982a). Changes in water content and volume accompanying exsheathment of *Haemonchus contortus*. *International Journal for Parasitology* **12**(1): 93-96.
- Davey, K.G. and R.I. Sommerville (1982b). Changes in optical path difference in the oesophageal region and the excretory cells during exsheathment in *Haemonchus contortus*. *International Journal for Parasitology* **12**(6): 503-507.
- Dehority, B. A. (2003). *Rumen Microbiology*. Nottingham, University Press.
- Desponsmiller D.D. and D.J. Jackson (1972). Actinomycin-D and puromycin-HCl in axenic culture of the nematode *Neoplectanum glaseri*. *Journal of Parasitology* **58**: 774-777.
- Eriksson, E.A. and A. Liljas (1991). X-Ray crystallographic studies of carbonic anhydrase isozymes I, II, and III. *The Carbonic Anhydrases: Cellular Physiology and Molecular Genetics*. S. J. Dodgson, R.E. Tashian, G. Gros, and N.D. Carter. New York, Plenum Press: 33-48.
- Khalifah, R.G. and D.N. Silverman (1991). Carbonic anhydrase kinetics and molecular function. *The Carbonic Anhydrases: Cellular Physiology and Molecular Genetics*. S. J. Dodgson, R.E. Tashian, G. Gros, and N.D. Carter. New York, Plenum Press.
- Nilsen, T.W. (1993). Trans-splicing of nematode premessenger RNA. *Annual Review of Microbiology* **47**: 413-440.
- Petronijevic, T. and W.P. Rogers (1986). Organic and inorganic acids as a stimulus for exsheathment of infective juveniles of nematodes. *International Journal for Parasitology* **16**(2): 163-168.
- Petronijevic T., W.P. Rogers and R.I. Sommerville (1985). Carbonic acid as the host signal for the development of parasitic stages of nematodes. *International Journal for Parasitology* **15**(6): 661-667.
- Petronijevic, T. and W.P. Rogers (1983). Gene activity and the development of early parasitic stages of nematodes. *International Journal for Parasitology* **13**(2): 197-199.
- Rogers, W.P. (1958). Physiology of the hatching of eggs of *Ascaris lumbricoides*. *Nature London* **181**: 1410-1411.
- Rogers, W.P. (1960). The physiology of infective processes of nematode parasite; the stimulus from the animal host. *Proceedings of the Royal Society of London. Series B. Biological Sciences* **152** (948).
- Rogers, W.P. (1962). *The Nature of Parasitism*. New York and London, Academic Press.

- Rogers, W.P. and F. Brooks (1976). Zinc as a co-factor for an enzyme involved in exsheathment of *Haemonchus contortus*. *International Journal for Parasitology* **6**: 315-319.
- Rogers, W.P. and R.I. Sommerville. (1960). The physiology of the second ecdysis of parasitic nematodes. *Parasitology* **50**: 329-348.
- Sommerville, R.I. (1954). The second ecdysis of infective nematode larvae. *Nature* **174**: 751.
- Sommerville, R.I. (1957). The exsheathing mechanism of nematode infective larvae. *Experimental Parasitology* **6**: 18-30.
- Spitzer, K.W., R.L. Skolnick, B.E. Percy, J. P. Keener and R. D. Vaughan-Jones (2002). Facilitation of intracellular H^+ ion mobility by CO_2/HCO_3^- in rabbit ventricular myocytes is regulated by carbonic anhydrase. *Journal of Physiology* **541.1**: 159-167.
- Tripp, B.C., S. Kerry, and J.G. Ferry (2001). Carbonic Anhydrase: New insights for an ancient enzyme. *The Journal of Biological Chemistry* **276**(52): 48615-48618.

CHAPTER 4

***OSTERTAGIA OSTERTAGI* CARBONIC ANHYDRASE PROMOTER CHARACTERIZATION AND PARTIAL GENOMIC ORGANIZATION**

4.1 INTRODUCTION

While carbonic anhydrases (CA) have been identified in virtually all organisms, those of mammalian have been most extensively studied. At least 14 distinct isozymes of CA have been identified in mammals. Eleven of the isozymes are active enzymes that catalyze the hydration of carbon dioxide and are essential for regulation of cellular pH and carbon dioxide transport (Sly 1995; Kim 2000). Each isozyme is classified by its temporal and spatial pattern of expression and amino acid composition.

CAIII is unique among the CA isozymes, as it has the slowest catalytic rate of the known CA's and possesses a phosphatase activity that can efficiently dephosphorylate phosphotyrosine residues (Cabiscol 1996). In mice, CAIII constitutes 8% of the soluble protein in red skeletal muscle (Carter 1991) and 25% of that of differentiated adipocytes (Spicer 1990). Interestingly, mouse CAIII (CAR3) expression in preadipocytes is negligible, but expression levels become substantial upon cell differentiation, through an unknown differentiation-dependent event (Kim 2000).

An additional characteristic of CAIII, as described in CAR3, is its ability to conjugate glutathione through disulfide linkage of its two reactive sulfhydryl groups, a process referred to as S-glutathiolation (Lii 1994). *In vivo* and *in vitro* studies have demonstrated that CAR3 is rapidly glutathionylated when cells are exposed to oxidative stresses, suggesting that the enzyme plays a role in responses to these stresses (Kim 2000).

CAIII is also highly developmentally regulated, with complex expression patterns occurring in the developing notochord, the primary axial structure of the embryo and in

developing muscle as soon as myotomes differentiate. Little is known, however, about the regulatory control of CAIII gene expression, although regulation of gene transcription is presumed to be the major determinant (Edwards 2000).

cDNAs encoding a CAIII have been isolated from the parasitic nematode *Ostertagia ostertagi* (OoCAIII). This parasite infects the bovine abomasum and is considered one of the most pathogenic and economically important nematodes of cattle. Entry of the infective third stage larvae (L₃) into the predilection site of the abomasum requires the removal of the retained second stage cuticle that encapsulates the L₃. This process, termed exsheathment, occurs in the bovine rumen through a mechanism that is postulated to involve a CA. Expression analysis of the OoCAIII in *O. ostertagi* L₃ revealed a greater than 4 fold increase following initiation of exsheathment in the bovine rumen. As a step towards understanding the physiological functions and biological significance of OoCAIII, the gene was isolated from *O. ostertagi* genomic DNA (gDNA) to analyze the primary structure, the exon-intron organization of the gene and 2kb of the 5' flanking region of the gene.

4.2 MATERIALS AND METHODS

4.2.1 Isolation of *Ostertagia ostertagi* Genomic DNA

A frozen pellet of 55,000 sheathed *O. ostertagi* L₃ was transferred to a stainless mortar, and ground to a fine powder in liquid nitrogen using a pestle. Grinding of larvae continued until only small fragments of larvae were observed by viewing a small aliquot of powdered larvae under a compound microscope and 100x magnification.

Powered larvae were subsequently transferred to a 1.5 ml microcentrifuge tube for isolation of genomic DNA (gDNA) using the QiAMP® Mini DNA isolation procedure and supplied reagents (Qiagen, Valencia, CA). Powdered larvae were suspended and mixed gently in

a volume of 180 µl ATL buffer. Twenty µl of Proteinase K mix was then added at the larval suspension and incubated for 1.5 hr at 56°C with gentle shaking in a hybridization oven. Proteinase K is an endolytic serine protease that cleaves peptide bonds at the carboxylic side of aromatic, aliphatic, and hydrophobic amino acids and thus greatly enhances protein digestion and denaturation of cellular organelles and the nuclear envelope.

Following Proteinase K digestion, 10 µl of an RNase cocktail was added to the sample, mixed by pulse-vortexing and incubated for 2 min at room temperature (25 -28°C). This step functions to remove RNA from the sample. A 200 µl volume of AL buffer was subsequently added to the sample followed by a 10 min incubation at 70°C. After the incubation period, 200 µl of 100% ethanol was added to the sample, mixed by pulse-vortexing, briefly centrifuged, loaded onto a QiaAMP spin column contained within a 2 ml microcentrifuge tube then centrifuged at 8,000 rpm for 1 min. The spin column contains a filter matrix that binds DNA while enabling digested proteins, RNA, and free nucleotides to be removed from the DNA sample. Following the centrifugation step, the column was transferred to a clean 1.5 tube and 500 µl of AW1 buffer was loaded onto the column, incubated for 1 min at room temperature and centrifuged for 1 min. The filtrate was discarded and the column was again transferred to a clean 1.5 ml centrifuge tube at which time 500 µl of AW2 buffer was loaded onto the column then centrifuged at 14,000 rpm for 3 min. Finally, the column was transferred to a clean 1.5 ml centrifuge tube and 200 µl of sterile distilled water was loaded onto the column, incubated at room temperature for 1 min and centrifuged for 1 min at 8,000 rpm. This step was repeated three times by reloading the eluate onto the column followed by centrifugation. This final step elutes the gDNA from the column matrix.

The DNA concentration was determined by analyzing a 1:15 dilution (60 µl volume; 56 µl water, 4 µl DNA) of the DNA in a spectrophotometer at 260nm. The DNA was visualized on a TAE agarose gel with ethidium bromide. This DNA extraction procedure resulted in a final yield of 122 µg of gDNA from the 55,000 sheathed *O. ostertagi* L3 which equates to 2.2 ng of gDNA / L₃.

4.2.2 Restriction Enzyme Digestion of *Ostertagia ostertagi* Genomic DNA

Four blunt-ended digested gDNA libraries, referred to as (DL1, DL2, DL3, and DL4) were constructed using the enzymes listed in Table 4.1. Digestion of gDNA was carried out by adding 8 µl of restriction enzyme and 10 µl restriction enzyme buffer to 2.5 µg gDNA. Sterile deionized water was added for a final reaction volume of 100 µl. This reaction was gently mixed by inverting the tube and incubated for 2 hr at 37°C. Following the 2 hr incubation, the sample was briefly mixed by vortexing then returned to 37°C and incubated for 16 hr. Following the digestion period, 5 µl of the digested DNA was visualized on a 1.2% agarose/ethidium bromide gel (Figure 4.1).

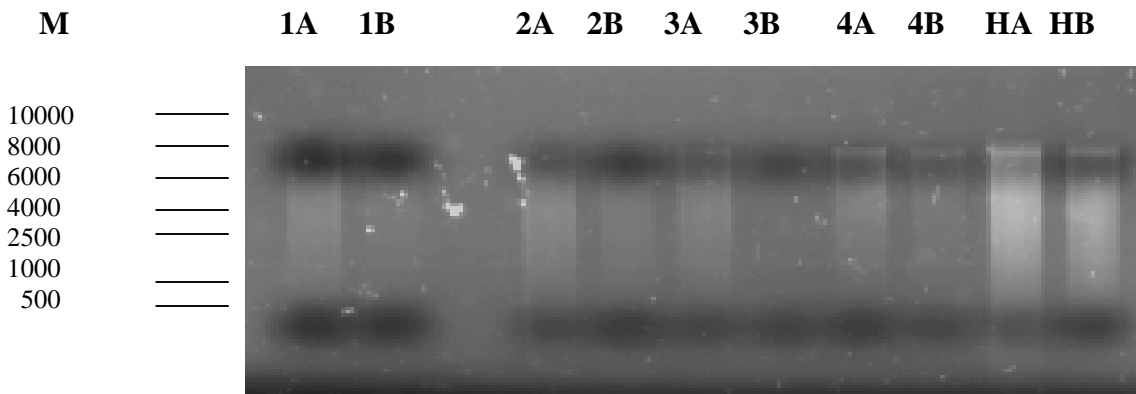


Figure 4.1. Agarose gel visualization of digested gDNA used in isolation of *Ostertagia ostertagi* CAIII promoter sequence. M, marker. 1, DL1 (*Pvu*II). 2, DL2 (*Dra*I). 3, DL3 (*Eco*RV). 4, DL4 (*Stu*I). H, human gDNA (control). A, digested, pre-phenol-chloroform cleanup. B, post phenol chloroform cleanup.

Table 4.1. Restriction enzymes used to produce digested genomic DNA libraries for use in the GenomeWalker protocol to obtain the promoter and non-coding regions of the *Ostertagia ostertagi* carbonic anhydrase gene.

RESTRICTION ENZYME	LIBRARY REFERENCE ID	RECOGNITION SEQUENCE
<i>PvuII</i>	DL1	CAG CTG
<i>DraI</i>	DL2	TTT AAA
<i>EcoRV</i>	DL3	GAT ATC
<i>StuI</i>	DL4	AGG CCT

4.2.3 Purification of Digested *Ostertagia ostertagi* Genomic DNA

Following the digestion procedure, 95 µl of phenol was added to each reaction tube and mixed by vortexing. The aqueous and organic phases of each sample were separated by brief centrifugation. The aqueous phase was recovered and transferred into a fresh 1.5 ml microfuge tube and the organic phase was discarded. To each tube, 95 µl of chloroform was added, mixed by pulse vortexing and centrifuged briefly to separate the aqueous and organic phases of the sample. The aqueous phase of each sample was then recovered and transferred to a fresh microcentrifuge tube and the organic phase was again discarded. To each tube, 190 µl or 2 times volume of the recovered aqueous layer of ice cold 95% ethanol, a 1/10 volume (9.5 µl) of 3 M sodium acetate (NaOAc) (pH 4.5), and 20 µg glycogen was added and mixed by vortexing. This suspension was centrifuged for 10 min at 15,000 rpm to pellet the gDNA. Following centrifugation the supernatant was decanted and the gDNA pellet was washed with 100 µl of ice cold 80% ethanol and centrifuged at 15,000 rpm for 5 min. The supernatant was subsequently decanted and the samples were dried in a speed vac at high heat for 5 min. The gDNA pellet was then resuspended in 20 µl of sterile distilled water. From each reaction 1 µl of sample was loaded

onto a 1.2% agarose/ethidium bromide gel for a qualitative determination of gDNA following purification.

4.2.4 Ligation of *Ostertagia ostertagi* Genomic DNA to GenomeWalker™ Adaptors

The Universal GenomeWalker™ DNA walking procedure (Clontech BD Biosciences, Palo Alto, CA) was used to isolate gDNA 5' of the start codon of the CA gene. This protocol is designed to find unknown gDNA sequences adjacent to a known sequence such as cDNA. The first step in this procedure is to construct pools of uncloned, adaptor-ligated gDNA fragments referred to as GenomeWalker™ libraries. The gDNA fragments were constructed by enzyme digestion as described in the previous section. The adaptor sequences ligated onto the gDNA are shown in Figure 4.2. Adaptors were ligated to each GenomeWalker library by adding 1.9 µl of GenomeWalker adaptor (25 µM), 1.6 µl of 10X ligation buffer, and 0.5 µl T4 DNA ligase (6 units/µl) to 4 µl of the digested purified gDNA. This reaction was carried out in a thermocycler for 18 hr at 16°C. The reaction was subsequently stopped by a 5 min incubation at 70°C after which 72µl of TE (10mM Tris, 0.1 mM EDTA, pH 7.5) was added to each reaction. These adaptors will provide a means to isolate sequences upstream of the CA start codon and non-coding regions within the CA gene.



Figure 4.2. The adaptor sequences ligated onto the ends of digested *Ostertagia ostertagi* genomic DNA for use in the GenomeWalker protocol for the isolation of the promoter region. Also shown are the sequences of adaptor primer 1 (AP1) and adaptor primer 2 (AP2).

4.2.5 Isolation of the Carbonic Anhydrase Promoter Sequence.

In order to isolate sequence upstream of the start codon of the OoCA gene, PCR reactions (walks) were carried out on each of the four digested GenomeWalker libraries. Each walk consisted of running a primary and secondary PCR reaction on each library, thus eight PCR reactions were carried out for each walk. The primary reaction was amplified with a CA specific primer and the adaptor primer AP1 (see Figure 4.1 for sequence) and the secondary reaction utilized a nested CA specific primer and the nested adaptor primer AP2 (see Figure 9 for sequence). For the primary reaction a PCR master mix was prepared by combining 1 µl of 50X Advantage Genomic Polymerase Mix (Clontech BD Biosciences, Palo Alto, CA), 5 µl 10X Tth PCR reaction buffer (Clontech BD Biosciences, Palo, Alto, CA), 2.2 µl Mg(OAc) (25mM), 1 µl dNTP (10mM each), 1µl of AP1 primer (10µM), 1 µl OoCA gene specific primer (10µM), 1 µl digested gDNA, and 37.8 µl deionized water. A PCR reaction was subsequently carried out on each GenomeWalker™ library with using thermocycler conditions of 7 cycles at 94°C for 25 sec and 72°C for 3 min followed by 32 cycles at 94°C for 25 sec and 67°C for 3 min. The reaction was completed with a 7 min extension at 67°C.

Upon completion, 1 µl of the primary PCR reaction was diluted in 49 µl of deionized water for use as template in the secondary PCR reaction. For the secondary PCR reaction, a PCR master mix was prepared exactly as described for the primary PCR master mix except nested primers were used for amplification and 1 µl of the 1:50 diluted primary PCR reaction was used as template. The PCR reaction was carried out in a thermocycler programmed to run for 5 cycles at 94°C for 25 sec and 72°C for 3 min followed by 20 cycles of 94°C for 25 sec and 67°C for 3 min. The reaction was terminated with a 7 min extension at 67°C.

The identification and respective sequences of all primers used to amplify the CA promoter region are listed in Table 4.2. All amplicons resulting from each walk were purified, cloned and sequenced exactly as described in Chapter 3 for the processing of cDNA clones.

The primary PCR for the first genomic walk used the OoCAR1 primer that was originally used to amplify the OoCA gene from cDNA, in conjunction with the AP1 primer. The secondary PCR reaction was amplified with the reverse complement of the OoCAF1, originally used to isolate the CA gene from cDNA and the AP2 primer was used as the sense primer. Four additional walks were carried out, one of which was necessary to recover sequence data from a clone that was too long to obtain in one sequencing event.

4.2.6 Isolation of the Genomic DNA Sequence of *Ostertagia ostertagi* Carbonic Anhydrase.

The Universal GenomeWalker procedure (Clontech BD Biosciences, Palo Alto, CA) was also used to isolate and sequence the genomic CA gene. The same four digested gDNA libraries used to isolate the promoter region were used here, as were the PCR conditions described above. Table 4.2 lists all primers used to obtain the CA gene. In brief, the primer pair used in the primary PCR reaction of the first walk was OoCASTRT that includes the start codon of the gene and was used in isolating the 5' of the CA gene from cDNA. The AP1 primer was used in conjunction with the OoCASTRT. For the secondary PCR of the first walk, the OoCAF1 primer was used as a nested primer in conjunction with the AP2 primer.

The reverse complement of the OoCAR1 primer was used in the primary PCR reaction of the second walk in conjunction with the AP1 primer. For the secondary PCR reaction a nested gene-specific primer was not used. Instead the OoCAR1 reverse complement primer was used with the AP2 primer. The primers CA3PM and AP1 and CA3PMx3 and AP2 were used for the primary and secondary PCR, respectively.

Genomic DNA sequence was also determined using Advantage 2 Taq polymerase on undigested gDNA. Reactions consisted of 50-100 ng *O. ostertagi* gDNA, 5 µl Advantage 2 10x PCR buffer, 1 µl each primer (at 10 µM), 1 µl dNTP (at 10 mM) and 1 µl Advantage 2 Taq. Reaction conditions included an initial denaturation at 95°C 1 min, then 25 – 30 cycles of 95°C 10 sec, 68°C 3-6 min. For cloning of the PCR products into T-tailed vectors a final extension for 10 min at 70° was included. PCR products were analysed by agarose gel electrophoresis.

Table 4.2. Primers used in the isolation of the genomic DNA sequence of *Ostertagia ostertagi* carbonic anhydrase III. Primers were used either in Advantage 2 Taq polymerase based PCR or with the Universal GenomeWalker kit to isolate the DNA sequence of the promoter region of OoCAIII or to determine the intron/exon structure of the gene.

Primer	Sequence (5'-3')	Protocol
STRTRC	gatgacctgagagttggctgagaaacgcat	Promoter isolation
PvuI5CA	cctaaccataaccatgctgtctaacctactggca	Promoter isolation
EcoRVpmgmwk	ccattctcgaagtgattaatacgcgcgtgaagcttgat	Promoter isolation
2CAPGAPF	acggcaaggttgatggcgaataaggg	Promoter isolation
2CAPGAPRC	gctattgattctagcgaggagtgaat	Promoter isolation
CASTRT	atggcggtttctcagccaactctcaggtcatc	Intron /Exon
CAF1	ctcgactcacttggaagtcctgcac	Intron /Exon
CAF2	ggagttggcgggtgcgtttctgcc	Intron /Exon
CaIntI3pmgw	ctggcgattgcgattttctgaacgaaagccatg	Intron /Exon
CAR1	gtcgtgtgactcattgagaaagatggcgat	Intron /Exon
CA3PM	ggcgtgggctatcgggcgaaactgcactctgattcatcgt	Intron /Exon
CA3x3	cacttggaagactgcacgtgtccgg	Intron /Exon
CA3x5RC	gcgatggatcagatgcagatgcagttcacc	Intron /Exon

4.3 RESULTS

4.3.1 Isolation of *Ostertagia ostertagi* CAIII Genomic Clones

Polymerase chain reaction using both the GenomeWalker technology and undigested gDNA produced numerous clones encoding for the genomic DNA construct of OoCAIII. Figure 4.3 shows an agarose gel depicting the size of some of these gDNA constructs. All of these clones have not been fully sequenced. However, based upon the sequence currently known, and upon the size of the fragments amplified with gene specific primers, a conservative estimation of the size and intron/exon structure of OoCAIII can be conducted.

Products obtained with three different primer sets are depicted in Figure 4.3. These products were generated in an attempt to determine the size of intron 3, which has not been fully sequenced. Amplicons generated with CA3x3+CA3x5RC (lanes 1 and 3) were approximately 2000 bp. Amplicons generated with CA3x3+R1 were approximately 2100 bp. The two primers CA3x5RC and R1 are 115 bp apart on the OoCAIII cDNA clone. The products generated with these primer pairs using cDNA as template are 301 bp and 406 bp, respectively. These data suggest approximately 1700 bp of intron exists between CA3x3 and CA3x5RC / R1. Only 2 introns exist between these primer sites, introns 3 and 4. Intron 4 is known to be 72 bp in length, suggesting intron 3 is approximately 1628 bp. Similarly, the amplicon produced using F1+CA3x5RC was slightly greater than 2000 bp (lane 5), while that produced with F1+R1 was approximately 2100 (lane 6). The primer F1 is positioned 14 bp upstream from CA3x3. Consequently, the amplicons produced with these primers appear similar in size. The sizes of these amplicons support the presence of an intron (intron 3) of approximately 1600 bp.

Figure 4.4 shows a schematic of the amplicons and their predicted sizes, based upon agarose gel electrophoresis. The full sequence is currently known for Exons 1, 2, 3 and 4, as evidenced

by closed boxes in figure 4.4. Additionally, the sequences of introns 1, 2 and 4 have also been determined. Intron 3 has not been fully sequenced to date. However, analysis of PCR products that have since been cloned and found to contain this intron, suggest the intron is approximately 1600 bp in length. Approximately 900 bp of this intron have been sequenced. The full-length cDNA sequence is known for OoCAIII and can be used to determine the minimum size of the OoCAIII gDNA clone.

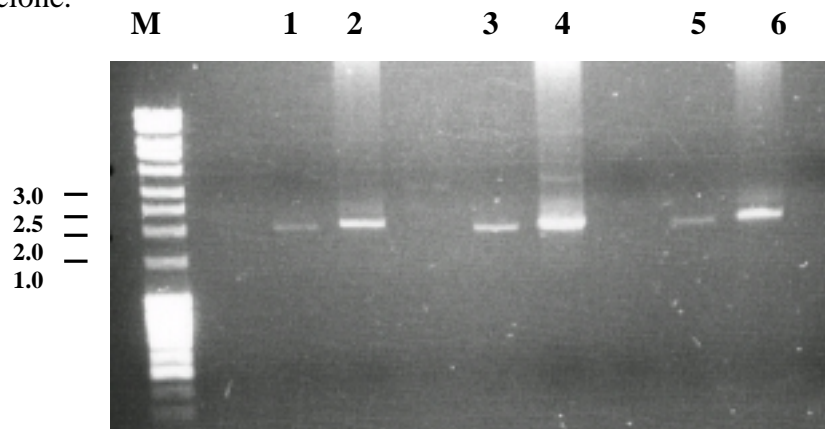


Figure 4.3. Electrophoretic analysis of amplicons isolated from *Ostertagia ostertagi* genomic DNA using primers specific to OoCAIII. M, marker. 1 and 3, CA3x3+CA3x5RC. 2 and 4, CA3x3 + R1. 5, F1+CA3x5RC. 6, F1+R1.

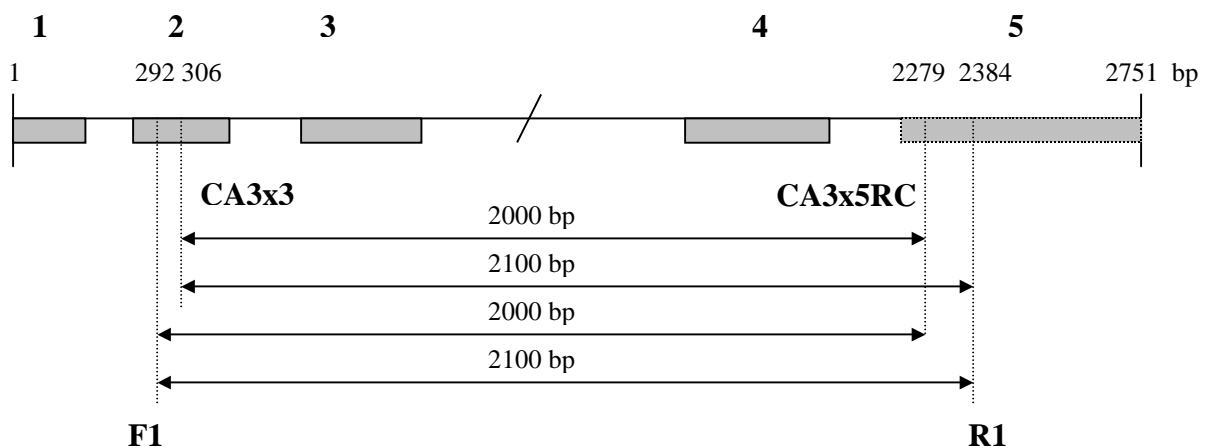


Figure 4.4. Schematic of amplicons obtained using OoCAIII-specific primers and *Ostertagia ostertagi* gDNA. The size of the amplicons suggests intron 3 is approximately 1600 bp in size. The boxes represent known exons. Single solid lines represent known introns. / = intron not fully sequenced. Dashed box represents unknown intron/exon structure.

4.3.2 Molecular Features of the *Ostertagia ostertagi* CAIII Gene

The full-length sequence of the genomic DNA encoding OoCAIII has not been determined. However, it is known that OoCAIII has at least 5 exons, encoding for 2247 bp of sequence (including the estimated 1600 bp intron 3). The fifth exon starts at 2266 bp. Analysis of the cDNA sequence encoding for this gene shows that, if no further introns are present, the fifth, and final exon, encodes for the last 165 amino acids, or 495 bp, making the genomic DNA sequence of OoCAIII 2751 bp. Figure 4.5 shows the intron/exon map of OoCAIII based upon currently known genomic DNA sequence. The currently known genomic DNA sequence of OoCAIII is presented in Appendix 2.

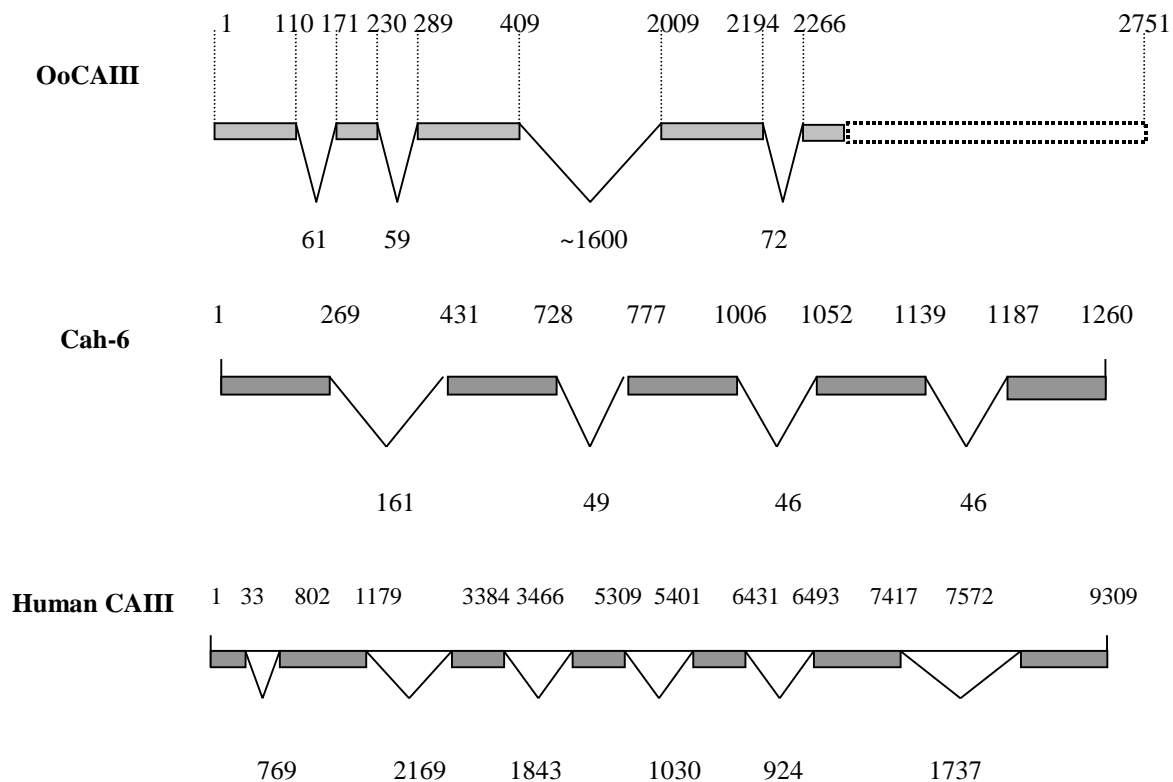


Figure 4.5. Schematic of genomic DNA structure of the *Ostertagia Ostertagi* CAIII (OoCAIII), *Caenorhabditis elegans* carbonic anhydrase cah-6 and human carbonic anhydrase 3 (HCAIII). Boxes, exons. Open lines, introns. Red box in OoCAIII indicates unknown intron/exon structure.

Nucleotide sequencing has shown that OoCAIII differs considerably from cah-6 in both size and intron and exon number and structure. Figure 4.5 shows a comparison of the genomic structure of OoCAIII with that of the *C. elegans* cah-6 and human CAIII. The genomic DNA sequence of cah-6 shows this gene only has 5 exons and is shorter (1260 bp) than OoCAIII (which appears to be at least 2751).

4.3.3 Characterization of the 5' Upstream Region of the *Ostertagia ostertagi* CAIII Gene

Three genomic walks 5' to the initiation codon of OoCAIII resulted in the isolation of 1,758 bases (Figure 4.6). The entire sequence was subsequently analyzed using Patch™ (www.gene-regulation.com) and Alibaba2 (www.alibaba2.com) transcription factor (TF) pattern analysis software to locate transcription factor binding sites within the sequence. Table 4.3 lists some of these regulatory elements.

A CAAT putative transcription initiation sequence was identified at nucleotides -55 and -77 from the start codon. Also, 13 potential *cis*-regulatory elements were identified within the promoter-proximal region (nucleotides -1 to -200). Most of the *cis*-elements identified in this region bind transcription factors that are known activators of genes associated with growth, differentiation or development. Some of these are listed in Table 4.3. Of particular interest, is the *cis*-element for the *Drosophila* pair-rule developmental related TF, Fushi tarazu factor (Ftz). *Cis*-elements for binding stress related TF's were also identified. These include binding sites for hypoxic transcription factors aryl nuclear translocator (ARNT) (at -1650) and a hypoxia inducible factor (Hif-1), identified at position -536. A heat shock response element was identified at position -1453 and an E-box motif beginning at nucleotide -536. The E-box motif is a DNA binding site for several transcription factors known to play roles in cellular growth and stress responses. Also, an upstream stimulatory factor (USF-1) was also identified beginning at

-1758

CTGAAGACAAGGTCTGACTTTTTGGTCGAACCTTAAGCTTTCTCAGAGAAATGCAGTACGCAAGGAGGA
GCACGTACACAATTGTGAGGATGATCTGACAGGAGCGAAARNTGCGTGAGGCCCTAATTGTGAGATGCAGCATT
GACCAACCTACAAATCAATGCTTTTCGCTTTATCGCTACTAAGGGATTGTGTATTTACCCGTGTTGGCGC
CATCATCCTGTTTTTTTAGTACTGACGTTACGCTTCACATCGCTGCAAGCGTGGTACCGCATTCTGTTAA
TCCCACACCCTACCTCTAGATHSE BindTAACCTCTAGAGTGGGGTAGAAACCAAAGGAAGTGCTGCTTAGTTTGCCG
GTAGATAAAGTTCCGGTCTTCACCGTTGTGTTCTTGTGAGCTGGCAAGGTTGGAGTTATGCACTTTCCTG
AAGGGGATTTTACGGCAAGGTTGATGGTGAATAAGGGGAAGTTTACCTAGTGGAAGAGAAGCGAAACG
ACAAAGGTGCGGACCGACAAGCAATTCGCGAAGGGTATGCCCCGGTGACCCAGCCGGCATTTCAGCGCCG
CTAGGTGCTGCCCCGCCCCTGACTGGCTACCCACTCGCCGTGAGTATTCATCCGTTTGCCAGAGAGAGC
GTAAGGTGAGAGGCGCGCAAATATGAGAGTCGCATCAATGGAATTAACGGGCAGGAAAGGTCCCATTTT
TTTTCTTCATTCCGCCATCTGTGACCACCTCAAACCTCTAATGAGCTCCTTCTAATCGTAAACTTCGCCTT
CAAAGAGAGACACTAGTGAGCCAGATTTAATCTGGCTTCTGATCGACTCGCAGATGGTAGTAGTCACAA
CCTCCCATCTTTTACAAGGTTTTCCTCTACTTGCTTTCCATTTACATAACATGACGTCGAAATCGGTGGATA
CTGTTGCGCCAAATAACTGTCCAAGCGTTGATAAGCTCTCCATGGAATTCAGGCGCGGCGCATTTCATGG
TCGACGATTGAGCCGAGCGTTATCGTTTGAATTTGACGAGACCTAGTCTGCCCCAAATTTCTGTGGATGCG
CTGCCGTCATACTCGCCCCCTTGAACATGGACTTGGCAATAACTGGAAGGCTAGCTGCCGTGGTAATGGTC
GCTCTTTGTGTTTCCTTGTGCTAGCCGTTTCTTCAACTCTTGTGTTTTACGCTTGCCCGTGATC
CTACTAGGGGCAAGGGAGCTACTATTCATAGAE-boxCACGTGCTGAATAAAGCCTCAAAAACTAATGCCTAACG
GGAGTGTGTCGAGGAGCATTCTGAGTGCTGAGTAACGATGAGTAAATGGACACCAGCTCGTTAAATATTT
HiF-1
GCTGAAATTTCACTCCTCGCTAGAAATCAATAGCAAACCACCTAAAAAGCGCTTTCGATCGGCTTCGTT
AGAAAGGGTCTACTGCGTTTTCAAGTAATCGCCTGGGTATTGGTTTCACAAATGGAAGATTGGCACCGC
TATTTGGCCACAAAGAACGTGTTTCTTGAAAGAATCGGCAGCAGAGTAAAGTAACTACGAATTCGGGG
GTTGTTTTTGCTACGATGACATCATCGGTGGCCAAAAGACGGAATCGGATCTCGCTCGCCTATGAATGCC
GCGAAATCTCTTCTCACTGCGAGCTTCCGTGAAAAATTCGTCCAGTTTTGGGACCAATCCAAGTGCTATA
CAAT box CAAT box
CAATAATTACTGCATCAAATAGCAATATTGCTGGCCAGATTATCAAGCTTCAGCGATCGTATTAATCA
CTTCGAGA *ATG*
-1

Figure 4.6. DNA sequence of the promoter region of OoCAIII showing some of the *cis*-regulatory elements reported. Start codon is in italics.

nucleotide -541. Additional binding sites for the stress activated TF's, activator transcription factor-3 (ATF-3), Elk-1, and fos were also very prevalent as were TF's associated with immediate early response gene expression.

Table 4.3 Binding sites for *cis*-acting elements found within the first 200 bp of the sequence immediately 5' to the OoCAIII. n=any, r=a/g, k=g/t, s=c/g, y=t/c, m=a/c, w=a/t..

Element	Recognition Sequence	Start position	End position
Ftz2	AAATAnyAAT	-62	-53
HNF-1	YyArTmATTA	-79	-70
C/EBP alpha	CCAATmAnTr	-79	-70
CREB	rTGACrTCAy	-204	-195
CRE-BP1	YnATGACrTC	-206	-197
C/EBP alpha	TrTTkGCTnA	-434	-425
Oct-1	ArATAywTGC	-437	-428
AP-1	YGCTGAskmA	-474	-465
c-Jun	YGCTGAskmA	-474	-465
USF	KrrwCACGTG	-541	-532
HIF-1	TACGTGCT	-536	-530
E-box	CACGTG	-536	-531
Sp1	GkGGCArGss	-563	-554
NF-1	MTTGGCmnTA	-679	-670
Sp1	GGsGsGGCGs	-797	-788
Cpc1	KAKKaGTCAy	-931	-922
CREB	nnrTGACGTC	-873	-864
CRE-Bp1	MmrTGACGTm	-873	-864

Table 4.3. continued

Element	Recognition Sequence	Start position	End position
C/EBP alpha	ywrCATAAsA	-880	-871
Oct-1	CATTkrsATA	-880	-874
Sp1	GyGGrGnCrG	-974	-965
NF kappa B	kGAAwnkyCC	-1075	-1066
NF kappa B	kGAAArkyCC	-1075	-1066
Sp1	rGAGGCGnGn	-1121	-1112
Sp1	CCsnCyCGCC	-1169	-1160
Sp1	nCCCGCCCms	-1189	-1188
Oct-2	nTATGCAAAT	-1235	-1226
ISGF	GGAAAnnG	-1287	-1278
NF kappa B	GGGAyTTymm	-1336	-1327
HSE-bind	AnmTTCkmGA	-1458	-1449
GR-alpha	AwCATmCTsT	-1548	-1539
ARNT	ynsnrnsTnGCGTGnnw	-1650	-1634

Several binding sites for Sp1, NF-kappaB, nuclear factor-1 (NF-1), a homologue of hepatocyte nuclear factor-2 (HNF-2), and CAAT/enhancer binding protein (C/EBP) were observed. Sp1 transcription factors are often associated with synergistic gene expression during differentiation. Also binding sites for steroid receptors, glucocorticoid, estrogen, progesterone and androgen were identified, as were 9 potential binding sites for octomer binding factor (Oct-1), an activator of hormone sensitive genes.

4.4 DISCUSSION

This work was based on the hypothesis that a CA is involved in the exsheathment of *O. ostertagi*. The isolation of OoCAIII has shown the existence of a CA in *O. ostertagi*. Furthermore, its pattern of expression suggests that while it may not be the molecule responsible for initiation of exsheathment, it appears to have a role during this developmental process. In order to more fully investigate the regulation of the expression of OoCAIII, the promoter region was isolated from gDNA and investigated for the presence of regulatory elements.

There are two classes of *cis*-acting elements, termed promoter and enhancer elements. Promoter elements are characterized by their dependence on position and orientation with respect to the transcription start site (McKnight 1986). The proximal promoter region consists of the first ~200 bases 5' of the start codon (Lodish 2001). Typical promoter elements are TATA, CAAT, and SP1, which are all involved in transcription initiation. Enhancer elements, however, are characterized by their ability to exert effects relatively independent of distance from, and orientation to, the transcription start site (Quinn 1988).

Analysis of 1,758 bases flanking the 5' end of the OoCAIII gene resulted in the identification of several potential *cis*-regulatory elements available for binding transcription factors. The majority of the TF binding sites identified within the promoter-proximal and distal-regions of the sequence are of the type associated with regulating genes involved in growth, differentiation, development, or adaptation to stress.

OoCAIII is highly upregulated in *O. ostertagi* L₃ larvae within 15 min of exposure to grass-derived rumen contents. The increased expression of OoCAIII mRNA coincides with initiation of exsheathment, a process that occurs in the rumen whereby the retained L₂ cuticle is removed. Exsheathment is required for larval development to the L₄ stage within the abomasum.

It has been postulated that initiation of exsheathment occurs as the larvae receive a stimulus or stimuli from its host (Sommerville 1957). Of the potential factors, a high partial pressure of CO₂ in a 37°C environment has been reported to be a very effective stimulus for initiating exsheathment (Rogers 1960). These conditions coincide with the environment within the bovine rumen, where normal temperatures are 38°C-40°C and normal CO₂ levels are 65% of the dissolved gases (Dehority 2003). *Ostertagia ostertagi* and perhaps other trichostrongylids, must have a means to adapt to heat shock and hypoxia while at the same time begin development to the first parasitic stage.

It is not surprising, then, to find *cis*-regulatory elements for the hypoxic induced transcription factors ARNT and HIF, as well as a heat shock response element within (HSE) promoter regions of the OoCAIII. In addition, an identified E-box motif and other *cis*-elements for environmental and mitogenic stress related transcription factors (such as ATF-3, USF-1, AP1, Elk-1 and c-jun) provide evidence for the occurrence of hypoxic and mitogenic stress signaling cascades during elevated OoCAIII expression and exsheathment.

It has been suggested that the host stimulus response for inducing exsheathment is transmitted from the host to the parasite *via* a CA within L₃ (Davey 1967). Based on mRNA expression levels relative to the exsheathment process, it does not appear that OoCAIII is the CA responsible for initiating exsheathment. The pattern of OoCAIII expression does, however, suggest a role in immediate downstream events following initiation and progression of exsheathment. The prevalence of *cis*-elements that potentially bind morphogenic, growth, and developmental TF's identified in the OoCAIII promoter regions further support this suggestion.

Within the first 100 bases of the promoter, *cis*-elements were identified that potentially bind Ftz, C/EBP, HNF-1, HNF-2, and SP1. Each of these TF's are known to regulate genes

required for cell growth and differentiation in metazoans (Holewa 1996; Slepak 2000; Zhang 2001; Reddy 2003). Ftz, for example, is a nuclear hormone receptor known to play a significant role in patterned gene regulation during early development of drosophila larvae. Perhaps more significant, a *C. elegans* homolog of Ftz, nhr-25 is required for embryogenesis, molting, vulval and gonadal development and RNA interference studies on nhr-25 produce an embryonic lethal phenotype (Brooks 2003). Thus, this TF seems to play a significant role in regulating genes involved in development.

Other developmental-related elements were also observed in the promoter region of OoCAIII. The C/EBP TF is critically involved in energy homeostasis and in regulating the balance between cell growth and differentiation. In addition, the occurrences of binding sites for NF-kappa TF were identified. NF-kappa is known to work synergistically with the helix-loop-helix leucine zipper (bZIP) family of transcription factors such as SP1, AP-1, ATF, c-fos, c-jun, C/EBP, and CREB in developmental and growth signaling cascades.

Other *cis*-elements identified suggest that OoCAIII is upregulated during a high physiological energy and growth state within the larvae and that this upregulation may be initiated in part by steroid hormone receptors. Evidence for regulation under a high energy and growth environment is provided by the identification of several cAMP response element binding proteins (CREB) and cell cycle related TF's such as E2-F and c-Ets-1. The potential for steroid control of the OoCAIII is supported by the identification of the *cis*-regulatory elements capable of binding estrogen, androgen, progesterone, and glucocorticoid steroid hormone receptors. Furthermore, the occurrence of Oct-1 TF's suggest OoCAIII is perhaps regulated in part by hormone receptor TF's as Oct-1 is often associated with genes that are sensitive to hormone regulation.

Although the entire genomic sequence of the OoCAIII has not been completely isolated before submission of this work, the complete coding sequence was obtained and is most homologous with the CAIII isozyme. As has been reported in the mouse CA3 and human CAIII isozyme, the OoCAIII appears to be developmentally regulated. This belief is based on the expression pattern observed in exsheathing larvae and is further supported by the putative *cis*-regulatory elements identified within the deduced promoter regions of the gene. The majority of these regulatory elements identified are known regulators of genes related to development, growth, stress, or differentiation. In addition *cis*-elements for TF's whose genes have been demonstrated to be upregulated by HIF-1 α , AP-1/c-jun, c-fos, ATF during hypoxic signaling were also identified within the promoter region of OoCAIII (Laderoute 2002).

Many PCR products have been generated from digested and undigested *O. ostertagia* genomic DNA using primers specific to the OoCAIII cDNA sequence. Based upon the sequences obtained to date, the OoCAIII genomic DNA clone appears to span at least 2751 bp. The gene encodes a minimum of five exons. The first four of these have been sequenced entirely from gDNA. However, it is unknown if the fifth exon is also the final exon, as occurs in cah-6. If it is the final exon, it must encode for 515 bp. This seems large, compared to the size of exons 1-4, which are between 100 and 200 bases in length. Human CAIII is actually encoded by 7 exons, and it appears likely that OoCAII may be similar. Further analysis of PCR products recently generated using OoCAIII specific primers on undigested gDNA may help resolve this question.

OoCAIII and Cah-6 also vary in that OoCAIII appears to be larger. A conservative estimate, based upon current knowledge, would place the gDNA for OoCAIII at 2715 bp. The

gDNA sequence encoding cah-6 is only 1260 bp. The presence of a 1600 bp intron in OoCAII, intron 3, accounts for much of this size difference.

In summary, this is the first report of a gDNA sequence encoding a CA from a parasitic nematode. While OoCAIII may be regulated in part by hypoxia, hypoxic conditions alone do not appear to be sufficient for increased expression of OoCAIII. In addition, analysis of the promoter of OoCAIII gDNA suggest its expression may be regulated by TF's associated with growth, development and /or differentiation.

4.5 REFERENCES

- Brooks, D.R., P.J. Appleford, L. Murray, and R.E. Isaac (2003). An essential role in molting and morphogenesis of *Caenorhabditis elegans* for ACN-1, a novel member of the angiotensin-converting enzyme family that lacks a metallopeptidase active site. *Journal of Biological Chemistry* **278**(52): 52340-52346.
- Cabiscol, E. and R. Levine (1996). The phosphatase activity of carbonic anhydrase III is reversibly regulated by glutathiolation. *Proceedings of the National Academy of Science* **93**: 4170-4174.
- Carter, N.D. (1991). Hormonal and neuronal control of carbonic anhydrase III gene expression in skeletal muscle. *The Carbonic Anhydrases*. S. J. Dodgson, R.E. Tashian, G. Gros and N.D. Carter. New York, Plenum Publishing Corporation.: 247-256.
- Davey, K.G. and S.P. Kan (1967). Endocrine basis for ecdysis in a parasitic nematode. *International Journal for Parasitology* **9**: 121-125.
- Dehority, B.A. (2003). *Rumen Microbiology*. Nottingham, University Press.
- Edwards, Y., F. Drummond and J. Sowden (2000). Regulation of the CA1, CA2, and CA3 genes. *The Carbonic Anhydrases*. W. R. Chegwidden, N. D. Carter and Y. H. Edwards. Birkhauser Verlag Basel, New Horizons.
- Holewa, B., E.P. Standmann, D. Zapp, P Lorenz, P. and G.U. Ryffel (1996). Transcriptional hierarchy in *Xenopus* embryogenesis: HNF4 a maternal factor involved in the developmental activation of the gene encoding the tissue specific transcription factor HNF1 alpha (LFB1). *Mechanisms of Development* **54**(1): 45-57.
- Kim, G., T. Lee, W. Anthony and R.L. Levine (2000). Nucleotide sequence and structure of the mouse carbonic anhydrase III gene. *Gene* **265**: 37-44.

- Laderoute, R. K., J.M. Calaoagan, C. Gustafson-brown, M. A. Knapp, G. C. Li, H. L. Mendonca, H. E. Ryan, Z. Wang. and R. S. Johnson. (2002). The response of c-Jun/AP-1 to chronic hypoxia is hypoxia-inducible factor 1 alpha dependent. *Molecular and Cellular Biology* **22**(8): 2515-2523.
- Li, C. K., Y.C. Chai, W. Zhao, J.A. Thomas and S. Hendrich (1994). S-thiolation and irreversible oxidation of sulfhydryls on carbonic anhydrase III during oxidative stress: a method for studying protein modification in intact cells and tissues. *Archives of Biochemistry and Biophysics* **308**: 231-239.
- Lodish, H., A. Derk, S.L. Zipusky, P. Matsudaira, D. Baltimore and J. Darnell (2001). *Molecular Cell Biology*. New York, W.H. Freeman and Company.
- McKnight, S. and. R. Tijan (1986). Transcriptional selectivity of viral genes in mammalian cells. *Cell* **46**: 795-805.
- Quinn, P.G., T.W. Wong, M.A. Magnuson, J.B. Shabb, and D.K. Granner (1988). Identification of basal and cyclic AMP regulatory elements in the promoter of the phosphoenolpyruvate carboxykinase gene. *Molecular and Cellular Biology* **8**(8): 3467-3475.
- Reddy, S.P., H. Vuong and P. Adisheshaiah (2003). Interplay between proximal and distal promoter elements is required for squamous differentiation marker induction in the bronchial epithelium: role for ESE-1, Sp1, and AP-1 proteins. *Journal of Biological Chemistry* **278**(4): 21378-21387.
- Rogers, W.P. and R.I. Sommerville (1960). The physiology of the second ecdysis of parasitic nematodes. *Parasitology* **50**: 329-348.
- Slepek, T.I., K.A. Webster, J. Zang, H. Prentice, A. O'Dowd, M.N. Hicks and N.H. Bishopic (2000). Control of cardia-specific transcription by p300 through myocyte enhancer factor-2D. *Journal of Biological Chemistry* **276**(10): 7575-7585.
- Sly, W.S. and P.Y. Hu (1995). Human carbonic anhydrases and carbonic anhydrase deficiencies. *Annual Review of Biochemistry* **64**: 375-401.
- Sommerville, R.I. (1957). The exsheathing mechanism of nematode infective larvae. *Experimental Parasitology* **6**: 18-30.
- Spicer, S. S., Z.H. Ge, R.E. Tashian, D.J. Hazen-Martin and B.A. Schulte (1990). Comparative distribution of carbonic anhydrase isozymes III and II in rodent tissues. *American Journal of Anatomy* **187**: 55-64.
- Zhang, C. K., W. Lin, Y.N. Cai, P.L. Xu, H. Dong, M. Li, Y.Y. Kong, G. Fu, Y.H. Xie, G.M. Huang and Y. Wang (2001). Characterization of the genomic structure and tissue-specific promoter of the human nuclear receptor NR5A2 (hB1F) gene. *Gene* **273**(2): 239-249.

SUMMARY

Presented in this dissertation are the results of efforts aimed at gaining an understanding of early developmental events in the representative trichostrongylid nematode, *Ostertagia ostertagi*, as it transitions from a free-living to a parasitic life stage. The basis of this work is to begin to identify potential targets for the development of novel pharmaceuticals aimed at controlling trichostrongylid nematodes in food animal species.

The specific objectives of this work were based on the hypothesis that the enzyme carbonic anhydrase (CA) has a significant role in the exsheathment process of trichostrongylid nematodes. This hypothesis was founded in the results of biochemical studies that provide strong evidence for the role of a CA in the exsheathment process of trichostrongylid species that infect sheep. However, at the time of initiation of the current work, a CA had not been reported from a trichostrongylid nematode.

Therefore, the specific objectives of this dissertation were to describe the kinetics of exsheathment of *O. ostertagi* in the bovine host, isolate a carbonic anhydrase enzyme from *O. ostertagi* and quantitate the expression of the enzyme during the time course of exsheathment. Finally, as a first step in understanding the potential role of the enzyme and how the enzyme is regulated, an analysis of the promoter region was conducted. Also, partial characterization of the genomic architecture of the gene was conducted to compare with CA's from other organisms.

Although the kinetics of exsheathment of several trichostrongylid nematodes have been conducted in sheep, such information has not been reported for *O. ostertagi* in cattle. Therefore, Chapter 2 describes the time course of initiation and progression of exsheathment of *O. ostertagi* following exposure to the bovine rumen. Early *in vitro* biochemistry studies on the exsheathment of trichostrongylid species in sheep demonstrated that a pH less than 6.0 significantly decreases

the kinetics of larval exsheathment in these species. Similar observations are presented in Chapter 2, where the initiation of exsheathment is more rapid when *O. ostertagi* larvae are exposed to a rumen environment with a pH of 6.0 or greater.

The mechanism for initiation of exsheathment is not known, but is postulated to be dependent on a high partial pressure of CO₂ and a temperature of 35°C or greater. The concentration of a reducing agent in the larval suspension and pH also affect the efficiency of the process. The enzyme carbonic anhydrase has been hypothesized as a primary factor in transmitting a host stimulus(i) to the parasite. CA carries out the reversible hydration of CO₂, yielding a hydrogen and bicarbonate ion. It is thought that the activity of CA increases the rate of CO₂ diffusion across the cuticle of the nematode. This rapid diffusion is hypothesized to result in a perturbation of intracellular pH, resulting in a neural endocrine signaling cascade that initiates exsheathment.

Chapter 3 presents the first report of a CA from a trichostrongylid nematode. A comparative analysis of the deduced amino acid sequence strongly suggests the enzyme is a CAIII isozyme and was so named OoCAIII. The pattern of quantitative expression of OoCAIII measured in *O. ostertagi* larvae exposed to an alkaline and acid rumen environment suggested that this particular enzyme was not responsible for initiation of exsheathment. However, the differential expression of OoCAIII observed in *O. ostertagi* larvae exposed to optimal exsheathment conditions, provides evidence for a role of OoCAIII in turning on or maintaining physiological processes that occur upon initiation and progression through the exsheathment process.

An analysis of approximately 1800 bp of sequence immediately flanking the 5' end of the OoCAIII transcription initiation site provides support for the role of OoCAIII in immediate early

events following exsheathment. This support is provided by the high prevalence of *cis*-regulatory elements that potentially bind transcription factors known to regulate genes involved in growth, development, differentiation, and stress adaptation. Of particular interest were the occurrences of binding sites for the hypoxia inducible transcription factors ARNT and HIF-1, and the presence of a heat shock regulatory element. Also several binding sites for transcription factors that have been shown to be upregulated by HIF-1 in mammalian systems were present in the promoter regions of OoCAIII.

CONCLUSIONS

The results of the work resulted in the recovery of the first carbonic anhydrase (CA) reported in a parasitic trichostrongylid nematode. The CA recovered was homologous to the human CAIII and *Caenorhabditis elegans* cah-6 isozyme. OoCAIII appears to be part of an early immediate response to exsheathment of *Ostertagia ostertagi* L₃. These results were obtained through the following objectives.

The first objective of this work was to establish an *in vivo* model to study the exsheathment process of *O. ostertagi* in the bovine rumen. Placement of L₃ into a disposable dialysis bag proved to be a successful approach as it provided a convenient means of studying early phenotypic and genetic developmental events following exposure to the environment of the bovine rumen. Access to the rumen was gained via a fistulated steer surgically fitted with a rumen cannula.

Two series of exsheathment experiments were conducted to determine the affect of pH on the rate of initiation of exsheathment of *O. ostertagi* L₃. The first series of studies were conducted while the steer was maintained on a 100% roughage diet on a paddock of abundantly available coastal bermuda grass. Measurements of rumen pH during this study ranged from 6.1 to 6.8. The second series of exsheathment studies were conducted while the steer was on a grain diet. While on this ration, measurements of rumen pH ranged from 4.8 to 5.5.

For L₃ exposed to grass-derived rumen contents, exsheathment was initiated within 15 min and at 30 min post-exposure, exsheathment had been initiated in 60% of the L₃. Two hr following exposure to the rumen, 98% of the L₃ had begun to exsheath. These observations are in sharp contrast to the exsheathment kinetics observed for L₃ exposed to grain-derived rumen

contents. Only 35% of L₃ had begun to exsheath after 1hr exposure to the rumen and maximum exsheathment (96%) did not occur until 6 hr post-exposure.

A number of parameters have been previously reported to affect the efficiency of exsheathment of the trichostrongylid nematodes *Haemonchus contortus* and *Trichostrongylus axei*. In addition to pH, CO₂, the concentration of a reducing agent, especially undissociated carbonic acid (H₂CO₃), reduction oxidation potential of the media and bicarbonate (HCO₃⁻) and salt concentrations can have significant effects. Although the only parameter measured in this study was pH, the magnitude of difference in pH values between the two studies has been demonstrated to affect the efficiency of exsheathment in reports of *H. contortus* and *T. axei*.

Biochemical studies aimed at understanding the physiological basis for exsheathment in *H. contortus* and *T. axei* provided strong evidence for the role of a carbonic anhydrase enzyme in initiation of exsheathment. Partial support for this hypothesis was the observation that *H. contortus* failed to undergo exsheathment when exposed to conditions conducive to exsheathment following incubation in the CA inhibitor ethoxzolamide. Based on the premise that CA activity is required for initiation of exsheathment, the difference in efficiency of exsheathment of *O. ostertagi* observed under both rumen pH conditions can be partially supported. Based on what is known about rumen physiology, the concentration of substrate molecules, HCO₃⁻ and H₂CO₃, for a CA would be much lower in grain-derived rumen contents in relation to grass-derived due to the significant decrease in salivation (a major source of HCO₃⁻) associated with this diet.

Although the evidence for the role of a CA is convincing, a CA, had not been isolated from a trichostrongylid nematode prior to the current work. Consequently, the second objective was to isolate a CA from *O. ostertagi* and subsequently measure the expression of the CA gene

in *O. ostertagi* L₃ during the time course of *in vivo* exsheathment. These efforts resulted in the successful isolation of a CA, named OoCAIII, as it was homologous to the human CAIII isozyme (HCAIII). OoCAIII levels in *O. ostertagi* L₃ increased 5 fold following 15 min exposure to grass-derived rumen contents. In addition, the expression pattern of OoCAIII in L₃ exposed to grass-derived rumen contents was very similar to the pattern observed for the percentage of L₃ undergoing exsheathment. In contrast, OoCAIII in *O. ostertagi* L₃ exposed to grain-derived rumen contents remained unchanged until 6 hr post-exposure when it increased 4 fold compared to unexposed L₃.

The expression pattern of OoCAIII observed in *O. ostertagi* L₃ during the exsheathment process suggests that this particular CA is not responsible for initiating the exsheathment process. This hypothesis is supported by the observation that L₃ exposed to grain-derived rumen contents continued to exsheath even though OoCAIII levels remained unchanged. Indeed, 90% of L₃ had begun to exsheath before OoCAIII levels increased.

It is well known that all CAIII isozymes analyzed in mammalian systems are resistant to inhibition by ethoxzolamide and other sulfonamide inhibitors. However, based on differences in the deduced amino acid sequence of OoCAIII, relative to human and mouse CAIII, there may be enough structural difference to render OoCAIII susceptible to sulfonamide inhibition. Alternatively, perhaps other CA's are functioning within *H. contortus* specifically or that trichostrongylids, including *O. ostertagi*, may possess other isozymes of CA as earlier reports of inhibition of exsheathment in *H. contortus* using ethoxzolamide suggest. To date 6 CA isozymes have been identified in the soil dwelling nematode *C. elegans*. Based on observations from this work, and in light of previously reported exsheathment studies in other trichostrongylids, it is not

unreasonable to suggest *O. ostertagi* may possess other CA's that may function to initiate exsheathment.

Although OoCAIII does not appear to be responsible for initiating the exsheathment process in *O. ostertagi*, it does appear to be upregulated under conditions conducive to efficient exsheathment. Perhaps it is associated with initiation or maintenance of immediate early events consequent to initiation of exsheathment. For example, in L₃ exposed to grass-derived rumen contents, initiation of exsheathment rapidly occurred and virtually all L₃ had begun to exsheath within 2 hr. Under these conditions expression of OoCAIII was quickly upregulated. For grain-derived L₃, initiation of exsheathment occurred without an apparent increase in OoCAIII expression although initiation was considerably slower to develop compared with L₃ exposed to grass-derived contents.

The CAIII isozyme in mammalian systems can be developmentally regulated and its expression is often associated with differentiation of cell types such as in adipocytes, myocytes, and glial cells. A possible explanation for the differential and transient expression of OoCAIII during exsheathment may be in response to a cellular signaling cascade initiated by the exsheathment process. Perhaps this putative cascade functions as a developmental "switch," signaling the L₃ to progress to the next developmental stage. The results of the promoter analysis suggest that OoCAIII transcription is highly dependent upon transcription factors that are known to activate developmental and hormone sensitive genes. Functional studies will help elucidate the role OoCAIII in *O. ostertagi* as it transitions from the free-living to the parasitic stage.

Many of the *cis*-elements identified in the promoter region bind transcription factor proteins whose genes have been reported to be highly upregulated through the hypoxic signaling protein HIF-1 α . This observation is interesting and suggests that OoCAIII is upregulated in

response to hypoxic conditions. Based on expression patterns observed in this study however, other regulatory elements are likely required for upregulation. This consideration is based on the observation that OoCAIII showed little to no increase in expression in L₃ exposed to grain-derived rumen contents although the rumen environment was theoretically hypoxic. The bovine rumen is normally 65% CO₂. Apparently OoCAIII expression is initiated in part by another TF or group of TF's.

Although the role of OoCAIII is not known, it appears to have a role either during initiation or following exsheathment. Awaited are studies aimed at describing the CO₂ hydratase activity of OoCAIII and the functional role this enzyme may serve in the transition of *O. ostertagi* L₃ from a free-living stage on pasture to a parasitic existence within the bovine host.

APPENDIX 1

OOCaIII FULL LENGTH TRANSCRIPT

ATGGCGTTTTCTCAGCCAACCTCTCAGGTCATTTTCAGACATTAAAGGAAAAAACAGCCACGATGGCTGAAG 70
 GAGCGCCCGCAATCGGAGCACATCTGCAGTCACTAAAAGTCGGTGCCCCAAAGTTGATCGAAAGTGC GTT 140
 GAATGCTAAAAACGGACTTCTCAATAGGGTGAAACTCGACTCACTTGGCAAAGTACTGCACGTGTCCGGT 210
 TTGGGTCGCTACCCATCACCTATTGATATCGTGCCGGTCATCACCTGCTTCGGAGAACATCTGCAAAATG 280
 CCGAGTTTCAGGGTCGAGTACCACAATACTGGCGACTTTTCGCGCAACGAATTCTGGCAACGCCGTCTGGCT 350
 GCTACGTGAAGGCAATGATTTCGGAGTTGGCGGTGTGTTTTCTGCCAGAGGAGCAGTATCATCTGGACTCA 420
 ATCACATGGCACTGGGGAACGGAGCCCATGAATGGCTCCGAACACACCATAGGAGGGGTTGGATACGCCG 490
 GCGAACTGCATCTGATCCATCGCAATACCCGCTTCCCGACGATGGAGTTGGCGCTGAAACAGCCGAACGG 560
 AGTGCTCGCGATCGCCATCTTTCTCAATGAGTCACACGACGAAAATCCAGCAATTACTCCGTTTATTGAG 630
 TTGTTACCGAATATCACCTACAAGGGCAACGAGGTCCGCGTCGGGCAGTTCAACTTCGGCGCACTTTTTTC 700
 CATCTCCAGAGAAGACTAAGGAGTTTTGGATGTACGAAGGATCGGAGACGGTAGAGCCTTTCCGCGAGAC 770
 AGTTCAGTGGCTTGTGTTCCGCTCAGCTGTGCCAATCAGCTCATATCAGCTTGATCGTCTACGTGAGGTT 840
 CGCTCTGGTGGCTACGACGAGGAGCGCGAGACACCGATGGAGCCGATTCTGTCCACCACAGCCACCAAAC 910
 CGCGCAGCGTCGTGTGTAGTTTTTCGCGAGCGCAGCCGGCGCTCCCGATCTTGGATTCAACAAACAGTAGcc 980
 ggccaggcgagtcggttcggctagcgatgagccctggcgctatccaccagccttggtaccacctgcttcc 1050
 cctttatcgctgcatgcgctcaccacctcagataacgcggttcagtcagtgctcgtctcacacacaact 1120
 gttttnattccataaaaaatccaagcgcatccttgacagatgttttcgaangatgaagtgtacagctgta 1190
 gcgtaggtcccttgcgcgagcaatatcttggtatcgTTTTTgcctatctgtaaaatgggatattccatt 1260
 gagaccctgaaatgcttacaagaagcgaagctttctgttttcaatgaaaatgtaagcaaataagtttcaa 1330
 atacgcttgcgccaacccggttcaccaatgatctcatcgtagtgctgctgttttctctacagaggcgaaa 1400
 tgttgccacaaacgctgacgcggcgcttttctcatcttatgtttatgtgcatactgttgccattccat 1470
 ttgtattctgtcgctttcacattctttctaataatcaccaattaattctttccgcggttggaagacacaa 1540
 gtgaagttcgaatcactcatgncattgtacaatatctgcaactcgtcaacgagctacgaangtagctcta 1610
 ttggcgataattttgttattttatcatttgatctcaattgctgatctccgccatgctaaccctcctatacat 1680
 aatgactgtggtaaatgtggttagacgatctctgaaatagatttataaaa~~aat~~aaaaagttccatagcaaa 1750
 aaaaaaaaaaaaaaaaaaaaaaaaaaaaaa 1777

Entire OoCaIII cDNA sequence. ATG, start codon. TGA, stop codon. Lowercase, 3' untranslated region. Green font, proposed polyadenylation signal and poly(A) tail signaling transcription termination.

APPENDIX 2 GENOMIC DNA SEQUENCE

1
ATGGCGTTTCTCAGCCTCTCAGGTCATCTTCAGACATTAAAGGAAAAACAGCCACGATGGCTGAAG
GAGCGCCCGCCATCGGAGCACATCTGCAGTCACTAAAAGTGAGATTGTTGCGTGGGTATTGATTTGTGGA
 TTCGATCAATCATGTCCGAAATTTAGGACCGGTGCCCCAAAGTTGATCGAAAGTTCGTTGAATGCCAAAA
TGGACTTCTCAATAGGGTAACTTTACAGGAGATCGGCTATTTGTCTTGGGTTCTGTTTAATTTGTTTT
 AGGTAAAACCTCGACTCACTTGGTAAAGTACTGCACGTGTCCGGTTTGGGTCGCTACCCTTCACCTATTGA
TATCGTGCCGGTCATCACCTGCTTCGGAGAACATCTGCAAAATGCCGAGTTTCAGGTGACTGGTTGAATGA
 AACAGCTTCGAATATGGGTATTCAGCAATCTATCAGAACACTAAAATAAAACACTAATCGATCAAACCTGA
 AACGTTCCCCTGGCGGTTCAATTTTTAGTGTGTACGGGTCGCACTAAAACACGGCTCATTTTTTGAAGGC
 TCCTGTCTCAGCGGCTAATTCTTGCCTTATCTGCTGTTTCTGAAATTACCTTTCTGTAATTTGAGTGTCTG
 TACATGTACATGGTTTCTATTTTGTCTGAGCCACGGAAGCCTCTCTCGTCAATCGGTAGCTATCGAC
 GGAAAGAGTGAGGATGGGCGGGTTTTCCAAAAAAGTGTGCAATTAGATGAACCCGATGAACCTTCGCTTC
 TTGAATTTACGAGCCTTGCAAATGTTGAGACCGGCACAAGTCAATTTCCATCACTAGAGGGACGTGGGCA
 ACTCGTGAATCCAAACACACCAGCTTGGTCAGTCATATGCACAGTAATTGAGAAAATTCAGA 902....
 (~731 bp unsequenced) ...1633 ATCCATACTCCCGCAAAACGTCTCGACTCG
 GTTTTATTGCGCTCTTGGATCGCGGAACGTTGATATGCTCAAGAACTGCGAGTTTCCCTCCCATAGCATT
 TAATCCCTTCCCCTTTTCTCCACCATTTCTAAAACTTGCTCAATTCAATGATAGCGAAAAACACTTTCA
 ATTAATGGATAAACAAATAAGGGTTCAGTGGTGCTCGTATCCACTTCAAGTGAATAAATCATTTTAGGGT
CGAGTACCACAATACTGGCGACTTTTCGCGCAACGAATTCTGGCAACGCCGTCTGGCTGCTACGTGAAGGC
AATGATTGAGAGTTGGCGGTGTCGTTTCTGCCAGAGGAGCAGTATCATCTGGACTCAATCACATGGCACT
GGGGAACGGAGCCCATGAATGGCTCCGAACACACCATAGGAGGTGGGTGTCTGGTTGTGCTGTTCTGACG
 TTAGGGAATGACGAATTTTCATTCAATTCATTCAATTCAGGGTTGGATACGCCGGTGAAGTGCATCTGATC
CATCGCAATACCGCTTCCCGACGATGGAGTTGGCGCTGAAACAGCCGAACGGAGTGCTCGCGATCGCCA
TCTTTCTCAATGAGTCACACGAC 2571

Known sequence of the genomic DNA encoding for *Ostertagia ostertagi* carbonic anhydrase III. Blue, underlined font represents exon sequence. Black, italicized font represents intron sequence. Dotted line indicates incomplete sequencing of intron 3. PCR suggests ~ 731 bp of sequence remains undetermined in intron 3.

VITA

Andrew Allan DeRosa, the brother of Donald F. DeRosa Jr. and son of Donald F. DeRosa Sr. and Shirley Ann DeRosa, was born in Longview, Texas, on June 16, 1967. He attended elementary school in Gulfport, Mississippi, and high school in Baton Rouge, Louisiana, until graduation in 1985. While attending Louisiana State University he earned a Bachelor of Science degree in the Department of Animal Sciences in 1992. On August 9, 1993, he began full-time employment in the Department of Veterinary Science as a research associate of Dr. James C. Williams in the ruminant parasitology laboratory. In the same year he began study in the Master of Science program of the LSU Department of Epidemiology and Community Health in the School of Veterinary Medicine. He completed the master of science program in the summer of 1997. While maintaining his full-time employment role in the Department of Veterinary Science, in 1998 he began the doctoral program of the LSU Department of Pathobiological Sciences in the School of Veterinary Medicine.